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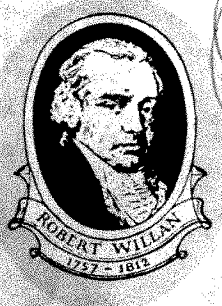

British Journal of Dermatology

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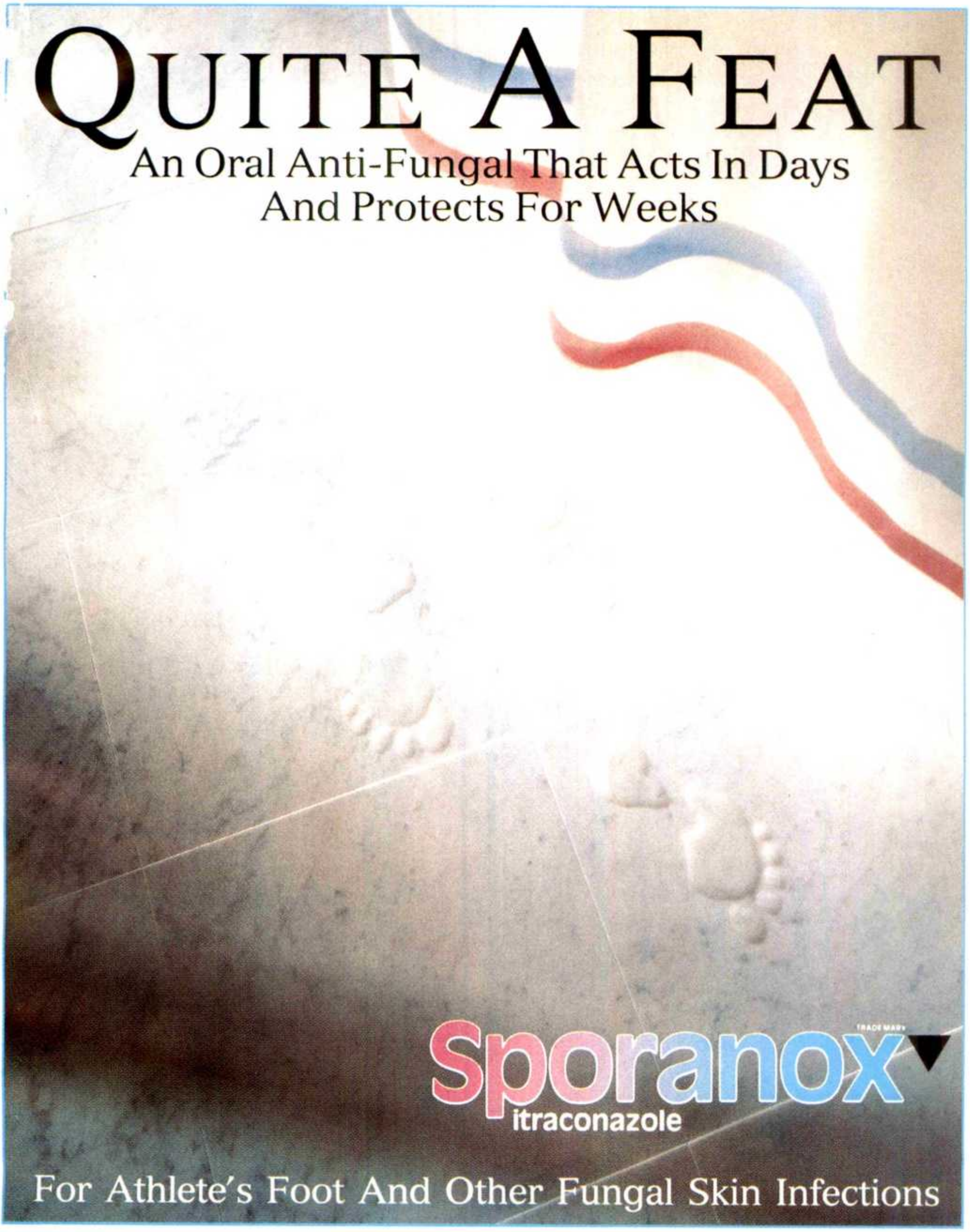
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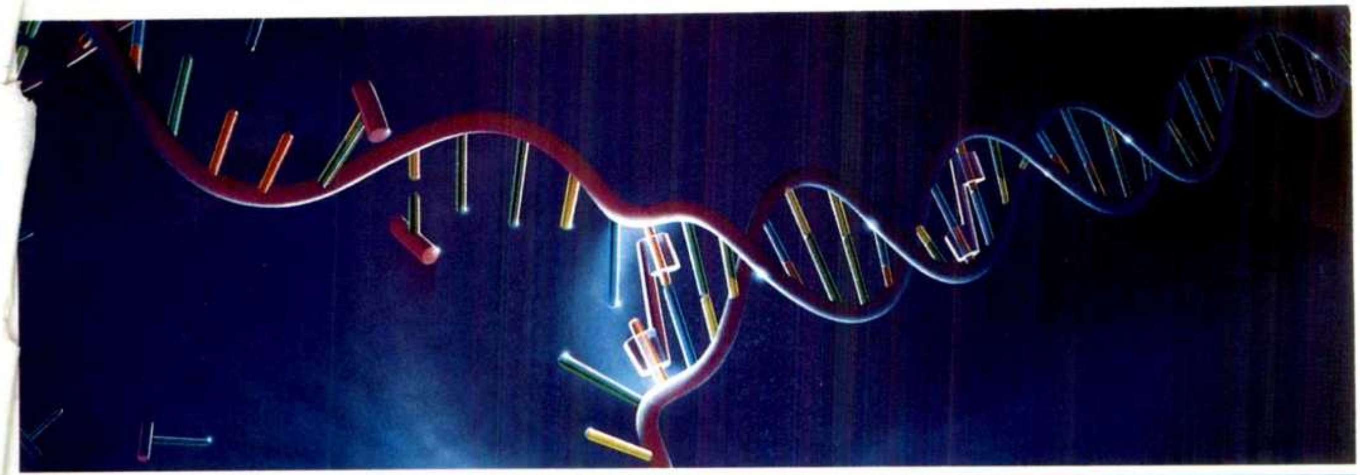

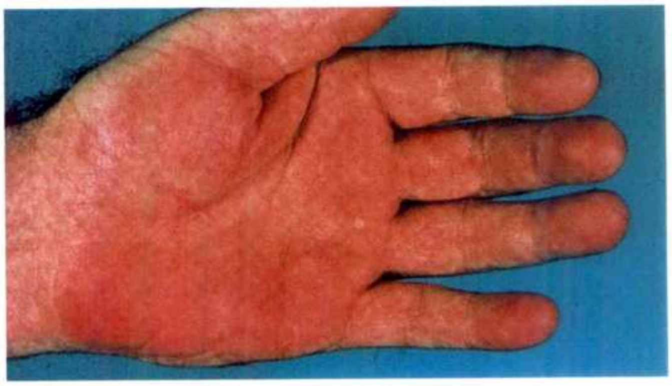

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[1] Edelson, R.L., Light-activated Drugs. Scientific American 259(2):68-75, August 1988.

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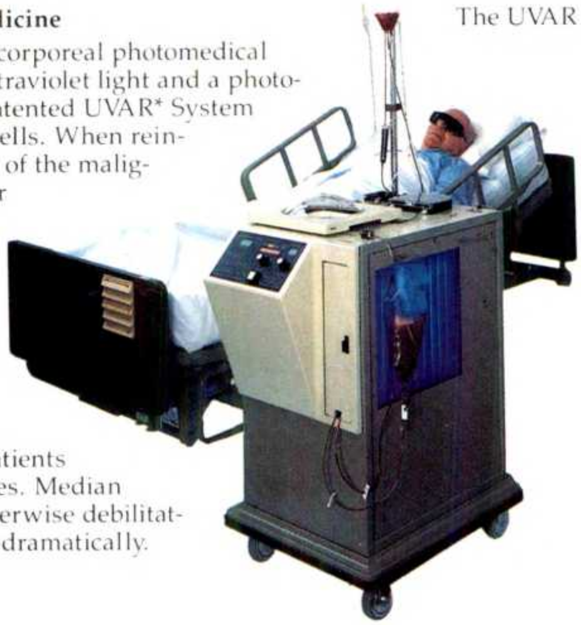

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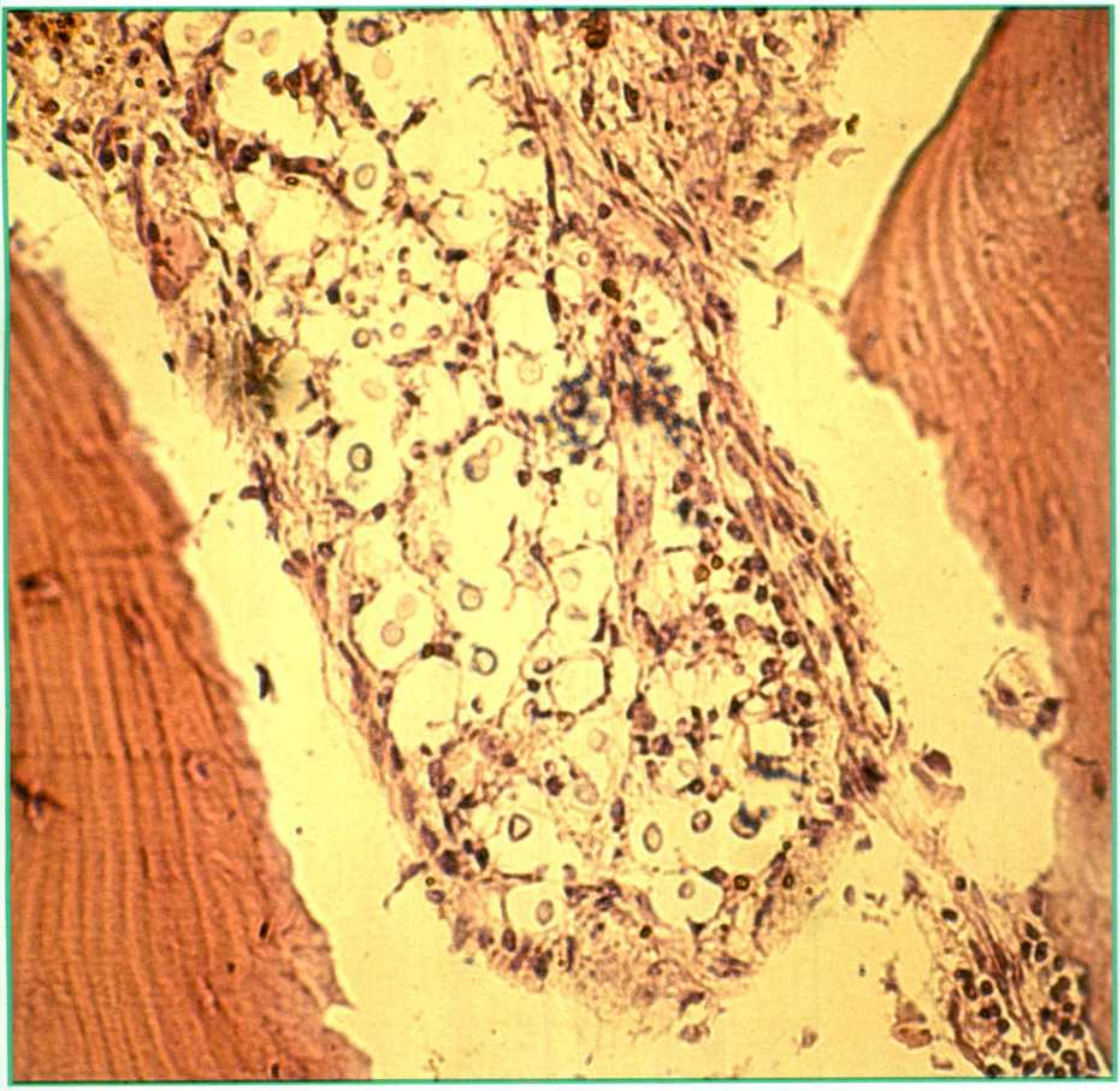

The journal MYCOSES provides an international forum for original papers, in English, on the pathogenesis, diagnosis, therapy, prophylaxis and epidemiology of fungal infectious diseases in man and animals as well as on the biology of pathogenic fungi.

Prominent mycoses of the skin have always been the domain of dermatology, whilst otorhinolaryngologists, gastroenterologists and gynaecologists are confronted with mycoses of the mucous membranes. Systemic mycoses are spread mainly over countries outside Europe, especially in the Americas, but in recent decades opportunistic, deep-seated, life-threatening mycoses in at-risk patients have become a world-wide challenge to medicine. Also, veterinarians have to deal with mycoses in domestic animals, some forms of which can be transferred from animals to man.

Medical mycology is a rapidly advancing field of medical microbiology. Effective therapeutic strategies are already available in chemotherapy and further strategies are being developed. Their application requires reliable laboratory diagnostic techniques which in turn result from mycological basic research. Opportunistic mycoses vary greatly in their clinical and pathological symptoms because the illness of the at-risk patient has a profound effect on their symptomatology and progress. MYCOSES publishes original articles on research work from all over the world reflecting current progress at the forefront of fundamental research and therapeutic development. Furthermore, highly informative case studies are published regularly. The short communications are of equal importance and interest, reporting on special mycological topics which make a valuable contribution to the field of fungal disease.

The journal MYCOSES is, therefore, essential reading for: scientists in fundamental mycological research, laboratory diagnosticians, clinicians in all fields (especially dermatologists, otorhinolaryngologists, gynaecologists, oncologists, haematologists, gastroenterologists and intensive-care specialists) and veterinarians.

Selection of recent and forthcoming articles:

T. Mochizuki, S. Tanaka, Y. Saito and S. Watanabe (Japan):
Mitochondrial Kinetics During Mitosis in Cryptococcus neoformans – an Ultrastructural Study

R. Kappe (Germany):
Coexistence of Free Antigens, Free Antibodies and Immune Complexes in Sera from Patients with Suspected Deep-Seated Candidosis

F. Staib and M. Seibold (Germany):
Use of the Membrane Filtration Technique and Staib Agar for the Detection of Cryptococcus neoformans in the Urine of AIDS Patients
– A Contribution to Diagnosis, Therapy and Pathogenesis of Cryptococcosis

R. Negroni, A. M. Robles, A. Arechavala and A. Tabordoa (Argentina):
Itraconazole in Human Histoplasmosis

A. N. Bulo, Suzanne F. Bradley and Carol A. Kauffman (USA):
The Effect of Cilofungin (LY 121019) in Combination with Amphotericin B or Flucytosine Against Candida Species

R. E. Herzog and Eva Ansmann (Germany):
Treatment of Vaginal Candidosis with Fluconazole

E. Guého, A. Bonnefoy, J. Luboinski, J.-C. Petit and G. S. de Hoog (France):
Subcutaneous Granuloma Caused by Phialophora richardsiae: Case Report and Review of the Literature

P. K. Shukla, M. Jain, B. Lal, P. K. Agrawal and O. P. Srivastava (India):
Mycotic Keratitis Caused by Phaeotrichoconis crotalariae. New Report

M. D. Richardson, G. S. Shankland and C. A. Gray (United Kingdom):
Effect of Amorolfine (Ro 14-4767/002) on in vitro Phagocytosis and Intracellular Killing of Candida albicans by Human Neutrophils

F. C. Odds and L. A. Merson-Davies (United Kingdom):
Colony Variations in Candida Species

M. J. Cunningham and W. C. Noble (United Kingdom):
SDS-PAG Protein Patterns of Yeasts from Human Sources

Annemarie Polak und A. Schaffner (Switzerland):
A New Experimental Model of Localized Candidosis for the Study of Antifungal Chemotherapy

Françoise Meunier, M. Gérard, V. Richard, L. Debusscher, H. Bleiberg and A. Malengrau (Belgium):
Hepatic Candidosis in a Patient with Acute Leukemia

T. Shimizu, M. Hiruma, M. Akiyama, A. Kukita and S. Tsuru (Japan):
Effect of Immunization and Potassium Iodide on Polymorphonuclear Leukocyte Chemiluminescence in Experimental Murine Sporotrichosis

A. Schaffner (Switzerland):
Experimental Basis for the Clinical Epidemiology of Fungal Infections. A Review

G. Caretta, F. Mancianti and L. Ajello (Italy/USA):
Dermatophytes and Keratinophilic Fungi in Cats and Dogs

R. Rüchel (Germany):
Identification of Certain False-Positive Results in the Cand-Tec® Test for Candidal Antigen

M. Mallié, B. Montès, J. C. Lebecq and J.-M. Bastide (France):
In Vitro Antifungal Activity of Saperconazole (R 66905) Against Candida and Torulopsis

F. Symoens, Ph. Willenz, V. Rouma, C. Planard and N. Nolard (Belgium):
Isoelectric Focusing Applied to Taxonomic Differentiation of the Trichophyton mentagrophytes Complex and the Related Trichophyton interdigitale

G. T. Cole, K. T. Lynn and K. R. Seshan (USA):
An Animal Model for Oropharyngeal, Esophageal and Gastric Candidosis

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EDITOR'S NOTE

A New Format

Readers will note that with this issue there has been a change in the format of the journal. The new size of 276 × 210 mm, known in the trade as truncated A4, will enable us to publish more papers, review articles, reports of meetings and abstracts. The acceptance rate of manuscripts submitted to our journal has dropped to 40%, despite the fact that over the years there has been an increase in the number of pages that have been published. It is with regret that many manuscripts that merit publication have had to be rejected because of lack of space. The change in format will enable us to print more articles, although there will be less pages in each issue. The increase in print will be about 20%. After 100 years the *British Journal of Dermatology* has recently made a number of changes and I hope that these are appreciated.

S.S.Bleehen

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REVIEW

Dermatology and the human gene map

CELIA MOSS
Department of Dermatology, Royal Victoria Infirmary, Newcastle upon Tyne NE1 4LP, U.K.

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Summary Chromosomal localization has been established for many genetic traits. Gene mapping may lead to the identification of disease genes, an understanding of pathogenesis, and the development of rational therapy, as well as facilitating antenatal diagnosis and genetic counselling. 'The new genetics'[1] is therefore of great interest to the clinician. Unfortunately the complex technology and unfamiliar vocabulary of molecular biology often deter non-specialists from keeping abreast of these developments. This account explains the principles of gene mapping, discusses its relevance to dermatologists, and lists the established loci of dermatologically important genes.

International Human Gene Mapping Workshops

The 10th International Workshop on Human Gene Mapping took place at Yale University in June 1989, with as many committees as there are chromosomes generating an 1100-page report.[2] Over 1600 genes had been localized, 419 in the 2 years since the 9th Human Gene Mapping Workshop. This includes a fair proportion of the 4344 known genetic traits,[3] although probably less than 2% of all the human structural genes. Dermatologists may be surprised to find that there are over 90 loci directly relevant to the skin (Table 1). Many more could be included such as those for oncogenes, complement components, T-cell receptors, cluster determinant antigens, cytochrome p450 enzymes, and immunoglobulins, and many more will have been added since. The International Human Gene Mapping Workshops are not to be confused with the American Human Genome Project, a systematic sequencing of the whole genome including the estimated 97% of non-coding DNA. The Human Gene Mapping Workshops are concerned primarily with the localization of functional genes: anonymous DNA segments are also catalogued but only for their use in further mapping of functional genes.

The motley assortment of diseases, enzymes and structural and other proteins whose genes have been mapped (Table 1) reflects the variety of methods used. To understand the scope and implications of gene-mapping, a grasp of some of the principles is necessary rather than a detailed knowledge of all the techniques.

Family linkage studies

Most of the diseases shown in Table 1 have been mapped by family linkage studies using a genetic polymorphism, which is a marker gene that can occur in different forms. In general, the closer two genes are on a chromosome the more likely they are to be transmitted together to offspring ('linked'), because the further they are apart the more likely they are to be separated during gamete formation (meiosis) when up to six recombination events or crossovers occur between each pair of maternal and paternal chromosomes. The way in which the degree of linkage is quantified is a source of great confusion, but can be understood in the following way. First, the presence or absence of the disease, and status with regard to the marker gene, is determined for each member of the family. Individuals may be dismissed as uninformative if a parent is homozygous for the marker gene so that it cannot be established whether or not recombination has occurred. If the two genes are widely separated, or not even on the same chromosome, on average half of the meioses will result in recombination between the disease gene and the marker gene and this is expressed as the recombination fraction, theta (θ), $=0{\cdot}5$. If the two genes are so close that they are never separated, $\theta=0$. Obviously the larger the family and the greater the number of informative meioses, the closer the observed proportion of recombinants will be to the 'true' recombination fraction, θ, for that pair of genes. Using tables or a computer program, different possible values of ranging from 0 to 0·5 are scored with regard to the

Table 1. Chromosome region: chromosomes are listed in numerical order and regions numbered from the centromere (cen) outwards along the short (p) and long (q) arms to the termini (ter). MIM number: the internationally accepted system for identifying individual genetic traits follows that set out in McKusick's Mendelian Inheritance in Man,[3] in which traits are categorized as autosomal dominant, autosomal recessive, or X-linked (the five-digit number beginning with 1, 2 or 3, respectively) and numbered alphabetically within these categories. Status indicates whether the localization is tentative (t), provisional (p) or confirmed (c)

Chromosome region	MIM number	Locus name (deficiency disease)	Status
1p	10940	Naevoid BCC syndrome	t
1p36	15560	Dysplastic naevus syndrome	p
1p34	17610	Uroporphyrinogen decarboxylase (PCT)	c
1q22-25		Endothelial adhesion molecule	p
1cen-q32	23371	Neutrophil cytosolic factor 2 (Chronic granulomatous disease autosomal 2)	p
1q21-22	14736	Involucrin	p
1q23-25	15324	Lymphocyte adhesion molecule 1	p
1q	13320	Erythrokeratoderma variabilis	p
1q	27870	XP group A	p
2q13-21	17686	Protein C (coumarin skin necrosis)	c
2q31-qter	13016	Elastin	c
2q31-32.3	12018	Collagen III α1 (Ehlers–Danlos syndrome IV)	c
2q34-36	22531	Fibronectin (Ehlers–Danlos syndrome X)	c
2q37	12025	Collagen VI α3	p
3p	19330	Von Hippel–Lindau disease	c
3p24	18022	Retinoic acid receptor β	p
3q28-29	15575	Melanoma associated antigen p97	c
4q12-21	17280	Piebald trait	t
4q21	15573	Melanoma growth stimulating activity	c
4q25	13492	Fibroblast growth factor (basic)	c
4q25	13153	Epidermal growth factor	c
4q28	13482,3	Fibrinogen A α and B β polypeptides	c
4q	26630	Hair colour (red)	t
4q	18160	Sclerotylosis	p
5q21.22	17510	Adenomatosis polyposis coli/Gardner's syndrome	c
5q31-32	13804	Glucocorticoid receptor	c
5q31.3-33.2	13491	Fibroblast growth factor (acidic)	c
5q31.3-33.2		Endothelial growth factor	c
6p21.3	21700	Complement component 2 (lupus systemic/discoid)	c
6p21.3	14280-93	Class I and II major histocompatibility complex	c
6q24-27	13343	Estrogen receptor	c
7p15	18697	T-cell receptor γ	c
7p13-12	13155	Epidermal growth factor receptor	c
7q21.3-22.1	12016	Collagen I α 2	c
7q21.3-22.1	16622	Osteogenesis imperfecta IV	p
7q31-32	21970	Cystic fibrosis	c
7q35	18693	T-cell receptor β cluster	c
8	13195	Epidermolysis bullosa simplex (Ogna)	p
9q	19110	Tuberous sclerosis 1	p
9q34	16120	Nail-patella syndrome	c
9q34	19350	Waardenburg syndrome	t
10q11.2	23370	Neutrophil cytosolic factor 1 (Chronic granulomatous disease autosomal 1)	p
11p15.4	11413	Calcitonin/calcitonin gene related peptide α	c
11p14.2-12	11416	Calcitonin gene related peptide β	c
11q12-13.1	10610	Complement component 1 esterase inhibitor (Hereditary angioedema)	c
11q12-13		Atopy	p
11q14-21	20310	Tyrosinase (oculocutaneous albinism)	p
11q21-22	22660	Collagenase (Dystrophic epidermolysis bullosa)	c
11q22-23	20890	Ataxia telangiectasia	p

Table 1 (*cont.*)

Chromosome region	MIM number	Locus name (deficiency disease)	Status
11q23-qter	17600	Porphobilinogen deaminase (acute intermittent porphyria)	c
11q	19109	Tuberous sclerosis 2	t
12p12.2-q11	12394	Keratin 4	p
12q12-13	15574	Melanoma antigen I	p
13q34	12013	Collagen IV α1	c
13q34	12009	Collagen IV α2	c
14q11.2	18688	T-cell receptor α	c
14q11.2	18681	T-cell receptor δ	c
14q	17620	Protoporphyrinogen oxidase (variegate porphyria)	p
15q13-15	10971	β2 microglobulin regulator	c
15q21-22.2	10970	β2 microglobulin	c
15q25-qter	12034	Collagen I α receptor	p
15	27876	Xeroderma pigmentosum group F	p
15		Retinoic acid binding protein 5	p
16q21	12036	Collagenase IV (basement membrane)	p
17p12-11		Keratin 14- and 16-related sequences	p
17q11.2	16220	Von Recklinghausen Neurofibromatosis (NF1)	c
17q12-21		Keratin 14- and 16-related sequences	p
17q21.1	18024	Retinoic acid receptor α	p
17q21.3-22	12015	Collagen I α 1 (Ehlers–Danlos VIIB)	c
17q21-23	14803	Keratin 15	p
17q21-23	23170	Keratin 19	p
19p13.3-13.2	14767	Insulin receptor (acanthosis nigricans)	c
19q13.2-13.3		DNA excision repair (one form of xeroderma pigmentosum)	c
19q	22724	Green/blue eye colour	p
19	14784	Intercellular adhesion molecule 1 (CD54)	p
19	11375	Brown hair colour	p
21q22.3	12022	Collagen VI α1	p
21q22.3	12024	Collagen VI α2	p
22q11-13.1	10100	Bilateral acoustic neuromas (NF2)	
Xp22.32	30295	Chondrodysplasia punctata	c
Xp22.32	30810	Steroid sulphatase (X-linked ichthyosis)	c
Xp21.1	30640	Cytochrome b-254 β polypeptide (Chronic granulomatous disease)	c
Xp11.4-11.21	30100	Wiskott–Aldrich syndrome	c
Xp11.21-cen	30830	Incontinentia pigmenti (sporadic)	c
Xcen-q13	30940	Menkes' disease	c
Xq12	31370	Androgen receptor (testicular feminization)	c
Xq12-13.1	30510	Hypohidrotic ectodermal dysplasia	c
Xq21.3-22	30150	α-galactosidase (Fabry's disease)	c
Xq25-27	30070	Albinism-deafness	c
Xq27-28	30500	Dyskeratosis congenita	c
Xq26-28	30831	Incontinentia pigmenti (familial)	c
Xq28		L1 cell adhesion molecule	c
Xq	30560	Goltz syndrome	p
X	30200	Epidermolysis bullosa, macular type	p
Yq11		Steroid sulphatase pseudogene	

possibility that this recombination fraction is consistent with the observed data. The logarithm of the odds for linkage at a particular value of θ is called the lod score, the term being derived from 'log odds'. The value of θ which gives the highest lod score is selected. In general, linkage is proven by a lod score greater than 3 (which means that, given this recombination fraction, the observed family data would be found by chance less than once in 10^3 times) and excluded by a lod score of less than -2. Taking the skin disorder erythrokeratoderma variabilis as an example, initially 29 genetic polymorphisms including eight blood groups, three immunoglobulin markers, six serum proteins, nine erythrocyte enzymes and three HLA antigens were determined in members of a large kindred.[4] Of these, only the rhesus blood group appeared to segregate with the disease: 26 of 27 affected individuals carried the rhesus genotype cde and the peak lod score was 5·72 at $\theta = 0{\cdot}03$.[5] In another family 17 out of 18 informative individuals with erythrokeratoderma variabilis carried the same rhesus genotype, while one had a different rhesus genotype and the maximum lod score was 4.21 at $\theta = 0{\cdot}03$.[5] Because the probability of linkage is expressed logarithmically the results from the two families can be combined by simple addition giving a final maximum lod score of 9·93 at $\theta = 0{\cdot}03$, with 95% confidence limits 0·008–0·11, meaning that the probability of recombination between the rhesus gene and the EKV gene is 3% (95% confidence limits 0·8–11%). Over short distances 1% recombination equals one unit of genetic distance known as the centimorgan, so the erythrokeratoderma variabilis gene is 3 centimorgans from the rhesus genes.

Genetic polymorphism can be observed not only in blood groups, enzymes, and other gene products but also in the DNA itself. Certain nucleotide sequences are selectively cut by particular restriction endonucleases, and variation in the number of such sequences results in so-called restriction fragment length polymorphisms (RFLP). Once a disease has been approximately mapped using biochemical polymorphisms it may be more precisely localized using RFLPs, as illustrated by tuberous sclerosis which was localized to the long arm of chromosome 9 by linkage with the ABO blood group,[6] and then more precisely mapped on the long arm of chromosome 9 using RFLPs.[7] Von Recklinghausen neurofibromatosis was localized by so-called exclusion mapping utilising multiple RFLPs, in an impressive international collaboration. At a meeting in 1987 set up by LINK, the neurofibromatosis association, unpublished negative linkage data from laboratories all over the world were pooled and an exclusion map was constructed which defined the last remaining candidate sites, so that efforts were concentrated on developing appropriate DNA probes and the precise position was soon established.[8] The DNA Committee of the 10th Human Gene Mapping Workshop which catalogues DNA polymorphisms recorded 1495 anonymous polymorphic DNA segments, as well as another 1922 anonymous DNA segments that are not polymorphic. Each new polymorphism mapped can serve as a marker for further localizations so it is not surprising that the rate of gene mapping is increasing so rapidly.

Family linkage studies are useful in genetic counselling. When a disease is closely linked to a polymorphic marker, the segregation of the marker with the disease within a particular family can be used to establish whether a foetus carries a disease gene, a procedure known as gene tracking.

Chromosomal rearrangements

Obviously establishing linkage between a disease and one or more polymorphisms can only be used to localize the disease when the position of one member of the linkage group has been ascertained. So how were the first genes localized? The sex-linked disorders were the first to have a chromosomal assignment, colour blindness being recognised as X-linked in 1911. The earliest autosomal assignments were made on the basis of gross chromosomal rearrangements. The first disease assigned to an autosome was Down's syndrome found in 1959 to be associated with trisomy 21. In 1968 Donahue *et al.* correlated in successive generations of Donahue's own family the segregation of Duffy blood group and an anomalous chromosome 1.[9] The development in 1970 of chromosomal banding made it possible to resolve smaller aberrations. In 1978 the ABO blood group was observed to segregate with an anomaly on chromosome 9,[10] leading to the localization of nail–patella syndrome, which had been found to be linked with ABO blood group 23 years previously.[11]

Chromosomal anomalies, which can now be recognized on the basis of rearrangements of DNA markers as well as banding, continue to provide important clues in gene mapping. Most genetic disorders are due to a deficient gene product so a deletion of part of a chromosome in a patient with a genetic disorder suggests that the relevant gene lies in the deleted segment. An observation of a patient with Gardner syndrome and a deletion on the long arm of chromosome 5[12] led to the localization of this disorder. The finding by Francke *et al.* of a small deletion on the short arm of the X chromosome in a boy with Duchenne muscular dystrophy, chronic granulomatous disease, retinitis pigmentosa and the McCleod blood group led to the localization of these 4 X-linked genes.[13] Chromosomal translocations, where chromosomes are broken and fragments transferred from one to another, are also extremely useful in gene mapping, because a break in a chromosome may localize a defective gene causing disease. It is not known whether the translocation is the cause or result of the gene defect. Evidence of a translocation with a breakpoint on the proximal short arm of the X chromosome in a girl with incontinentia pigmenti was the first clue to the localization of this disorder and has been confirmed in several other cases.[14] Translocations affecting the X chromosome are especially interesting because they may affect the process of X chromosome inactivation. In every female cell one of the two X chromosomes is inactivated so that females have the same 'dose' of X-linked genes as males, and whether the maternal or paternal X chromosome remains active is random (Lyonisation). However, in a female with a translocation affecting one X chromosome the other, normal X chromosome is preferentially inactivated. Thus, in all her cells the abnormal X chromosome will be active, and she may therefore manifest fully a recessive X-linked disorder which normally affects only males. A possible reason for this apparently disadvantageous phenomenon is that if the translocated X chromosome were inactivated, the inactivation process would spread to the autosome to which it was attached, with even more serious consequences as all autosomes carry essential genes. Anhidrotic ectodermal dysplasia[15] and Duchenne muscular dystrophy[16] are examples of X-linked diseases normally only seen in males, where an affected female with a translocation has helped to localize the gene.

Deletions and translocations are useful not only for localizing but also for identifying disease genes. A breakpoint can provide a useful starting point for sequencing a gene by chromosome-walking techniques. Patients with von Recklinghausen neurofibromatosis (NF1) associated with a translocation at the NF1 locus on chromosome 17 may well provide the opportunity to sequence this gene.[17]* Royer-Pokora *et al.*[18] cloned the chronic granulomatous disease gene using cells from the previously mentioned patient with an X chromosome deletion, using a novel technique of subtractive hybridization. They combined cDNA extracted from leukaemic cells (chosen as a likely source of the gene whose absence causes chronic granulomatous disease) with RNA from the patient with chronic granulomatous disease and a deletion: the unpaired cDNA contained the chronic granulomatous disease gene. This was probably the first example of reverse genetics, that is identifying a gene product on the basis of its map position.

Direct methods of gene mapping

In most of the examples mentioned so far, the product of the affected gene was unknown. Many different and ingenious techniques have been developed for localizing genes whose product or function can be identified. In principle these all combine a method of separating out the chromosomes and some test for the presence of the gene or its product. Somatic-cell hybridization was first developed by Weiss and Green to localize thymidine kinase.[19] They fused human cells with mouse cells lacking this enzyme, and noted that the hybrids progressively lost human chromosomes, and therefore thymidine kinase activity, with time, but that thymidine kinase activity was retained while one particular human chromosome, subsequently identified as chromosome 17, remained in the cell. Somatic cell hybridization has been used in over 80% of autosomal assignments. Other rodents may be used but in all cases human chromosomes are lost preferentially, and a panel of hybrid clones is used to correlate the presence of a particular fragment of human chromosome with the presence of a particular cell phenotype. Skin-related genes localized in this way include those for collagens, enzymes involved in porphyrin metabolism and xeroderma pigmentosum group A. A more recently developed method of separating chromosomes involves staining them with a fluorescent dye and sorting in a fluorescence activated cell sorter. *In situ* hybridization is perhaps the most direct method of gene-mapping: radiolabelled cloned DNA segments are hybridized to chromosomes separated in metaphase spreads and the gene locus revealed by autoradiography. This technique was used to localize more precisely the gene for the keratinocyte protein involucrin, once it had been mapped to chromosome 1 by somatic cell hybridization.

Genetic heterogeneity

It has been known for some time that genetic disorders may have more than one locus, for example there are both X-linked and autosomal forms of chronic granulomatous disease and retinitis pigmentosa. Advances in gene mapping have increased the number of disorders which appear to have genetically distinct but phenotypically similar subtypes. For example, tuberous sclerosis, initially mapped to chromosome 9,[7] now appears in some families to have a different locus.[2] The incontinentia pigmenti locus mapped by identifying the breakpoint on the X chromosome in sporadic cases due to translocations[14] does not correspond to the locus established from linkage studies in affected families.[20] The demonstration of genetic heterogeneity is forcing us to re-analyse the clinical data. Sporadic patients with incontinentia pigmenti due to a translocation are more severely affected, particularly neurologically, than familial cases. In the case of neurofibromatosis, gene mapping has clearly differentiated von Recklinghausen disease (NF1) on chromosome 17 from bilateral acoustic neuroma (NF2) on chromosome 22,[8] although there is phenotypic overlap. There is no example yet in dermatology of phenotypically different diseases resulting from different mutations of the same genes, as in the haemoglobinopathies, and Duchenne and Becker muscular dystrophies.

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* Note added in proof: this has now been achieved.[23]

Dermatologists and gene mapping

Why do dermatologists need to know about gene mapping? Firstly, so that we know which familial disorders are amenable to antenatal diagnosis and genetic counselling. Some diseases such as epidermolysis bullosa and Fabry's disease can be diagnosed antenatally without recourse to DNA technology. However, where the gene product is unknown and carriers may be phenotypically normal, as for example in tuberous sclerosis, or when diagnosis is required before the disease phenotype is detectable, gene tracking using closely linked DNA markers may facilitate both antenatal diagnosis and informed counselling of unaffected relatives. Secondly, genetic mapping of a disorder may help establish its cause. Close linkage between a disease and a gene coding for an abnormal protein suggests the pathogenesis, as in the case of Ehlers–Danlos type IV which was tightly linked to the type III collagen gene.[21] Furthermore, mapping of a disease can lead directly to sequencing and cloning of the relevant gene and identification of the protein responsible for the disease. This process of 'reverse genetics' has established the role of dystrophin in Duchenne and Becker muscular dystrophies and cytochrome b-245 in X-linked chronic granulomatous disease, and may soon reveal the molecular basis of genodermatoses such as anhidrotic ectodermal dysplasia, incontinentia pigmenti, tuberous sclerosis and neurofibromatosis. For many other multisystem inherited disorders, this seems the most promising approach to the pathogenesis. Thirdly, mapping and cloning disease genes opens up the possibility of gene therapy where a missing gene is inserted into a relevant cell line which is then reintroduced into the patient. This has recently been achieved in severe combined immunodeficiency disease, where the gene for the missing enzyme adenosine deaminase can be inserted, via a retrovirus, into the patient's T cells which are then cultured *in vitro* and injected back into the patient.

Finally, dermatologists have access to a wealth of valuable clinical material which could contribute greatly to our understanding of disease. We need to look again at our 'odd' patients, those with syndromes that do not quite fit a particular diagnosis. A patient with an autosomal dominant disorder but no family history might represent a new mutation, and cytogenetic studies could reveal a deletion or breakpoint identifying the relevant gene. Manifestation of an X-linked recessive disorder in a female may be due to a translocation. Association of a known genetic disorder with unusual features may reflect deletion of contiguous genes, for example some patients with X-linked ichthyosis are hypogonadal because the steroid sulphatase gene deletion extends to the adjacent locus for Kallman's syndrome.[22] Dermatologists cannot afford to ignore the dramatic and exciting advances in genetics, which, for the benefit of our patients, we should be prepared to contribute to and exploit.

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Distribution of skin-derived antileucoproteases (SKALP) in the marginal zone of the spreading psoriatic lesion

P.C.M.VAN DE KERKHOF, L.H.C.M.KUPPENS, Y.VAN VLIJMEN AND J.SCHALKWIJK

Department of Dermatology, University Hospital Nijmegen, Javastraat 104, 6524 MJ Nijmegen, The Netherlands

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Summary Two new elastase inhibitors (SKALP, skin-derived antileucoproteases) were recently described in the lesional skin in psoriasis. The present study investigated the distribution of SKALP activity in the marginal zone of spreading psoriatic plaques. In a 4-mm zone immediately adjacent to the erythematosquamous plaques, SKALP activity was slightly increased compared to distant uninvolved skin. Within the lesion the anti-elastase activity was pronounced, but was significantly higher in the central zone of the plaque compared to the periphery. The appearance of SKALP in the psoriatic lesion appears to be a late event compared to endothelial involvement, intraepidermal accumulation of PMNs, epidermal proliferation and abnormal keratinization. This observation lends further support for the hypothesis that the induction of anti-elastase activity is associated with the off-switch of cutaneous inflammation.

The intraepidermal accumulation of polymorphonuclear leucocytes (PMNs) and epidermal proliferation are well-established features of psoriasis. Molecular events which explain both phenomena may be of significance in the pathogenesis of this disorder. In this respect PMN-derived neutral proteinases and their inhibitors have been suggested to be of particular interest, as these proteolytic enzymes have been shown to accelerate epidermal cell-turnover and to enhance the accumulation of PMN in skin.[1,2]

Elastase and cathepsin G are serine proteinases derived from the PMNs. A repertoire of different inhibitors is present systemically or locally in tissues, to prevent damage by these enzymes: alpha-1-proteinase inhibitor (α-1-PI) and antileucoprotease (ALP).[3–5] The significance of these inhibitors as a self-defence system against serine proteinases has been shown in the lung and cartilage.[6–8]

Recently, several observations suggest that anti-elastase activity is induced in human skin in response to inflammation and injury.[9–11] Partial purification of this anti-elastase activity in psoriatic scales revealed two unique species (approximate molecular weights 10 and 20 kDa) which are heat-stable, high-affinity human leucocyte elastase inhibitors.[11] Analogous to the bronchial ALP we have suggested the acronym SKALP (skin-derived antileucoprotease) for these inhibitors.[11]

In this study we investigated at which stage of development of the psoriatic lesion SKALP activity is induced. Elastase-inhibiting activity was quantified in the marginal zone of the spreading psoriatic lesion, which is a practical *in-vivo* model[12–14] to study the transition from distant clinically uninvolved skin to the fully developed lesion.

Methods

Subjects, leukotriene B_4 application and biopsy procedure

Six patients, three females and three males, aged between 30 and 74 years, participated in this study. All patients had a chronic plaque psoriasis of many years duration and had not used any form of therapy during the previous 2 weeks. Their lesions were in a state of exacerbation indicated by a pronounced erythema, the appearance of pinpoint papules and a history of increased scaling or spreading of the lesions during the previous 2 weeks.

Aliquots (10 μl) of 20 ng leukotriene B_4 (LTB_4) (Paesel, F.R.G.) dissolved in ethanol were applied to a test area (5·5 mm diameter) of the clinically uninvolved skin immediately adjacent to the lesion.[15] After 24 h razor-blade biopsies were taken[10] and the amount of PMNs determined by measuring elastase activity (six patients) and by histological assessment (four patients).

Keratotome biopsies were taken at those sites in the marginal zone where the clinical edge was sharp. The boundary was carefully marked with a pencil. After cooling the skin surface with an ethyl chloride spray, a slice of approximately 1 × 2 cm was cut across the margin of the plaque, using a Castroviejo keratotome, set at a depth of 0·4 mm. The slices contained a clinically involved and uninvolved part of about equal sizes. Each slice was subdivided in eight sections, 2-mm-wide, four from the clinically involved and four from the uninvolved skin. The sections were rinsed in phosphate-buffered saline, dried between filter paper and weighed. Sections were stored at -80°C until analytical processing.

Biopsies were taken free handed from the LTB$_4$-treated areas using a razor blade in conjunction with a metal guard. Biopsies were snap frozen and stored at -80°C until processing.

Assay of elastase and SKALP activity in epidermal extracts

Biopsies were homogenized in an all-glass Potter grinder in extraction buffer containing 100 mM Tris, 1 M NaCl, 0·4% cetrimide, pH 8·5 as described previously.[10] Elastase activity was measured in all biopsies as described previously[8] using the fluorogenic substrate methoxysuccinyl-alanyl-alanyl-prolyl-valyl-aminomethylcoumarin (Bachem, Bubendorf, Switzerland). Inhibition of elastase activity by skin extracts was routinely measured as the percentage inhibition of a standard amount of elastase. A sensitive microassay[9] was used to allow measurement of small amounts of inhibitory activity. Briefly, 10 μl containing 1 ng leucocyte elastase was mixed with skin extract (10 μl) and incubated at 37°C for 15 min to allow complexing of enzyme and inhibitor. Thereafter, 20 μl of 250 μM substrate solution was added and the reaction was allowed to proceed for 30 min at 37°C. All components were dissolved in extraction buffer as indicated above. The incubation was terminated by the addition of 1 ml buffer (100 mM Na$_2$CO$_3$, pH 10·5) and fluorescence was measured at 375 nm (excitation) and 440 nm (emission) in a Perkin–Elmer LS-5 fluorimeter. Inhibition of elastase activity in this assay was calculated as the percentage inhibition of the activity of 1 ng elastase. One unit of inhibitory activity was defined as the amount required to reduce the activity of 1 ng elastase in this assay by 50%. Elastase activity was linear during the assay (final substrate concentration 125 μM, of which less than 10% is consumed during the 30 min incubation).

For the histological assessment of PMN, cryostat sections were stained with anti-elastase (Serotec, Oxford, U.K.) using a peroxidase technique.

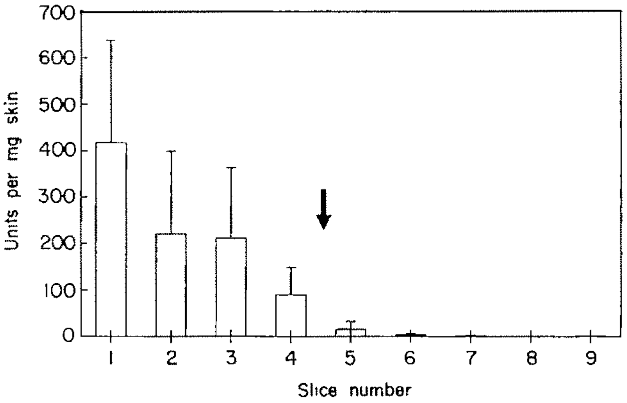

Figure 1. SKALP activity (mean ± SD) in different sections of the margin zone of six psoriatic lesions. The arrow indicates the border of the plaque.

Results

SKALP activity

SKALP activities in different sections of the marginal zone of the spreading lesion are shown in Figure 1. At more than 4 mm outside the clinical boundary all subjects showed SKALP activities of less than 4 units per mg.

There was a statistically significant decrease in the amount of SKALP activity from the centre of the lesion to the uninvolved skin outside the plaque ($r=0\cdot455$, $P<0\cdot005$, regression analysis). Also, within the lesion the decrease of SKALP activity from the centre towards the edge was statistically significant ($r=0\cdot282$, $P<0\cdot02$, regression analysis). There was a statistically significant effect of the position of the section in the biopsy on the amount of SKALP activity ($P<0\cdot0001$, Kruskal–Wallis test).

LTB$_4$-induced PMN accumulation

The presence of PMNs was investigated in the clinically uninvolved skin adjacent to the lesion, both enzymatically and histologically, following epicutaneous application of a 20-ng aliquot of LTB$_4$. Interestingly, a discrepancy was observed between the two methods. In none of the skin extracts ($n=6$) could PMN elastase be measured (data not shown). However, in three out of four biopsies that were examined histologically, PMNs could clearly be demonstrated either scattered in the dermis and epidermis or in microabscesses.

Discussion

The marginal zone of the psoriatic lesions showed similar dynamics with respect to SKALP activity in all six subjects. SKALP activities at more than 4 mm outside the edge were within the range of SKALP activities as observed in biopsies from normal skin.[9,11] Slightly, but significantly increased SKALP activities were observed between 0 and 4 mm outside the edge of the lesion compared to the distant clinically uninvolved skin and a more substantial increase was seen within the boundary, with a fourfold difference between the most peripheral and more central zones.

Comparing the dynamics of SKALP activity in the marginal zone of the lesion with the dynamics of markers for epidermal proliferation, keratinization and inflammation indicates that the induction of SKALP is a relatively late phenomenon. In a previous investigation marker enzymes for various skin compartments were measured in the margin zone of spreading psoriatic plaques.[12–14]

The release of elastase from the PMNs has been suggested to be relevant for the migration of these cells into the skin,[2] and it may be that SKALP is of relevance for the inhibition of PMN migration. Compared to the rather modest presence of PMNs in the central zones of the lesion the peripheral rim contains a relatively high number of PMNs.[15–17] Thus, the present study further supports the hypothesis that the presence of a pronounced SKALP activity inhibits transcutaneous migration of PMNs. In an earlier study it was shown that epicutaneous application of 100 ng of leukotriene B_4 (LTB_4) to distant uninvolved skin and paralesional skin caused a normal invasion of PMNs, whereas the LTB_4-induced PMN accumulation in involved skin was very sparse.[18] The present study showed that the application of a relatively low dose of 20 ng of LTB_4 on to the paralesional skin (sections 5–6) induced an accumulation of PMN which could be assessed histologically. However, the presence of SKALP in the paralesional skin (sections 5–6) is the most likely explanation for the fact that no elastase activity was measured at these LTB_4-treated sites, as extraction of the biopsy allows complexing of elastase with inhibitor present in the tissue. The distribution of SKALP over the margin of the psoriatic lesion agrees with the concept that anti-elastase acts in the skin as an off-switch for inflammation. The SKALP activity in the lesion may be an attempt to stabilize the psoriatic process; however, the relatively modest increase in the most peripheral zones of the lesion might permit a significant migration of PMN which then results in the spreading of the lesion.

Acknowledgments

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Tenascin expression in hyperproliferative skin diseases

J.SCHALKWIJK, IVONNE VAN VLIJMEN, BRIGIT OOSTERLING, C.PERRET,
R.KOOPMAN, J.VAN DEN BORN* AND ELEANOR J.MACKIE†
*Departments of Dermatology and *Nephrology, University Hospital Nijmegen, The Netherlands*
†Department Forschung, Kantonsspital, CH-4031 Basel, Switzerland

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Summary The expression of tenascin, a recently discovered extracellular matrix glycoprotein, was studied by immunohistochemistry in normal human skin and in a number of skin diseases with epidermal hyperproliferation such as psoriasis, basal cell carcinoma, Bowen's disease and solar keratosis. Tenascin expression in the upper dermis of normal skin was found to vary from almost absent to patchy along the basal membrane. Staining was continuous and intense around blood vessels, hair follicles and eccrine sweat ducts. In basal cell carcinoma a marked expression of tenascin was found in the tumour stroma, especially adjacent to the basal membrane surrounding the tumour cell nests. In Bowen's disease and solar keratosis, tenascin expression was found in the dermis next to the keratinocytes. In psoriasis the dermal papillae of clinically involved skin were intensely stained and a continuous band of tenascin was present in the upper dermis along the basal membrane. The distribution of tenascin differed from other known extracellular matrix components.

Tenascin is an extracellular matrix glycoprotein[1–4] which is identical to hexabrachion,[5] myotendinous antigen,[6] cytotactin[7] and glioma mesenchymal extracellular matrix antigen.[8] Its distribution suggests that it is important in the development of various tissues such as mammary gland,[9] hair follicles,[9] muscle,[6] teeth,[2] kidney,[10] cartilage and bone,[3] neural crest and derived tissues.[11] In addition, tenascin is re-expressed in connective tissue adjacent to epithelial malignancy in the mammary gland. The molecule is structurally distinct from other extracellular matrix glycoproteins such as fibronectin and laminin and its expression is subject to different control mechanisms.[4] cDNA clones mapping the cytotactin/tenascin/hexabrachion gene[12–14] revealed that there is homology to fibronectin, fibrinogen and epidermal growth factor. Tenascin is a disulphide-linked hexamer. For human tenascin various subunit molecular weights have been described, ranging from 225 to 320 kDa for a large subunit[14 15] and 150–180 kDa for a smaller subunit.[14] It was suggested that different forms of tenascin could be generated by alternative splicing, depending on the tissue where it is expressed.[12–14,16]

At present little is known of the function of tenascin. It was shown to promote cartilage differentiation *in vitro*[3] and it supports the growth of primary tumour cells in serum-free culture medium.[9] Tenascin can partially inhibit the attachment of cells to fibronectin,[17] but appears to act as a surface adhesion molecule in the central nervous system.[18]

Recently, the presence of tenascin in rat[4] and human skin[15] was reported. In normal human skin tenascin was detected diffusely in the subepidermal dermis and near the basement membranes of sweat glands. In rat skin tenascin was detected in a discontinuous distribution in the basement membrane of the dermal–epidermal junction. In addition it was detected in basement membranes of subepidermal capillaries and around hair follicles. In wound healing processes in rat skin, a marked increase of tenascin expression was found, particularly beneath the migrating and proliferating epidermis. *In vitro* experiments suggest that proliferating epithelium induces the production of tenascin by mesenchymal cells.[4] We therefore studied the expression of tenascin in human skin diseases characterized by epidermal hyperproliferation.

Methods

Antisera

Antiserum against tenascin purified from rat embryo fibroblast cultures was raised in rabbits as previously

Correspondence: Dr J Schalkwijk, Department of Dermatology, University Hospital Nijmegen, Javastraat 104, 6524 MJ Nijmegen, The Netherlands

described.[4] The serum was absorbed on a fibrinogen-Sepharose 4B column and its specificity was checked by Western blot analysis and enzyme-linked immunoadsorbent assay. No reactivity against fibronectin and fibrinogen (to which there is homology[12-14]) could be detected. The antiserum was found to react with human tenascin produced by Saos-2 cells (a tenascin-producing human osteosarcoma cell line).

In addition, a rabbit antiserum against tenascin from chick fibroblasts was used. The details of specificity have been published elsewhere.[1] Both antisera yielded identical staining patterns in all tissues examined.

A monoclonal antibody (JM-72) to heparan sulphate core protein was raised using human glomerular basement membrane to immunize BALB/c mice. Spleen cells were fused with Sp2/0 cells according to standard protocols. Ascites fluid was used at a 1:500 dilution.

Antilaminin serum (a gift from Dr J. Cleutjens, Department of Pathology, University of Maastricht, The Netherlands) was obtained by immunizing a rabbit with mouse laminin from Englebreth–Holm–Swarm mouse tumour cells. The antiserum was found to cross-react with human laminin.

Monoclonal antibody to human plasma fibronectin was obtained from Sigma Chemical Co, Mo, U.S.A. (clone F7387, ascites fluid). Peroxidase-conjugated antisera to mouse and rabbit immunoglobulins were obtained from Dakopatts, Glostrup, Denmark.

Tenascin extraction

A preparation containing human tenascin was obtained from conditioned medium from confluent Saos-2 cells. High molecular weight compounds were obtained by 40% ammonium sulphate precipitation followed by dialysis, giving a 100-fold concentration of the starting material.

Western blot analysis

Ammonium sulphate precipitates from culture medium or tissue extractions were dissolved in sample buffer (reducing conditions) and subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli[19] using a 5% gel. Proteins were blotted on nitrocellulose according to Towbin *et al.*[20] After incubation with antitenascin serum, the blots were treated either with alkaline phosphatase- or horse radish peroxidase-conjugated anti-rabbit immunoglobulin, for visualization of tenascin.

Patients

Biopsies were taken either from volunteers or from surgical specimens. Permission was obtained from the local ethical committee for experiments on humans. Punch biopsies (3 mm diameter, 5 mm deep) or shave biopsies (5 mm diameter, 0·3 mm deep) were taken after local anaesthesia. Tissue specimens were snap frozen in liquid nitrogen and stored at −80°C until they were used. Seven shave biopsies and three punch biopsies were taken from normal skin, eight from the back of the patients and two from sun-exposed areas on the face. This group included five men and five women, mean age 38 years (range 29–65 years). The psoriasis group consisted of eight patients (four men and four women; mean age 47 years, range 30–67 years). Biopsies (five shave and three punch biopsies) were taken from the back (seven patients) or from the leg (one patient). Both lesional and uninvolved skin were studied. Biopsies of basal cell carcinomas from six patients were taken from sun-exposed areas of the face in five and in one from the trunk. The mean age was 60 years (range 53–65). The solar keratoses (from two patients) and the lesions of Bowen's disease from three patients, were obtained from surgical specimens.

Histology

Cryosections were prepared on a Reichert Cryostat at −28°C. Sections (5 μm) were placed on bovine-serum-albumin-coated slides, fixed in acetone for 10 min and stored in sealed boxes at −80°C until used. In addition sections were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 30 min. If necessary, sections to be stained for immunoperoxidase were immersed in absolute methanol with 0·3% hydrogen peroxide to block endogenous peroxidase. Some sections were treated with bovine testicular hyaluronidase (0·1 mg/ml) in acetate buffer pH 5·5 for 30 min at room temperature as indicated for tenascin staining in rat skin.[4] Immunoperoxidase staining was performed as follows: after blocking of the endogenous peroxidase the sections were incubated in PBS for 5 min, then the slides were incubated with antisera diluted in PBS, 1:100 for antitenascin, 1:50 for antifibronectin, 1:50 for antilaminin and 1:500 for antiheparan sulphate proteoglycan (60 min at room temperature for all antisera). After washing in PBS sections were incubated either with peroxidase-conjugated rabbit anti-mouse Ig or swine anti-rabbit Ig (1:100 dilutions in PBS with 5% normal human AB serum), 30 min at room temperature. After washing the sections were developed using aminoethylcarbazole as a chromogenic substrate. After washing in PBS the sections were either counter-stained with haematoxylin or directly mounted in Kaiser's gelatin solution according to standard procedures.

Results

Using rabbit anti-rat tenascin serum followed by immunoperoxidase staining, tenascin expression was examined in normal and pathological skin samples of human adults. The antiserum used was shown to cross-react with human tenascin. A Western blot of the high molecular weight fraction of conditioned medium of Saos-2 cells is shown in Figure 1. The molecular weight of human tenascin was estimated to be 330–340 kDa, using reduced (250 kDa) and unreduced (500 kDa) human fibronectin and myosin (200 kDa) as marker proteins. This value is in agreement with the observation of Lightner *et al.*[15] Previously published values, obtained by extrapolation from Biorad high molecular weight standards, are probably too low.[15]

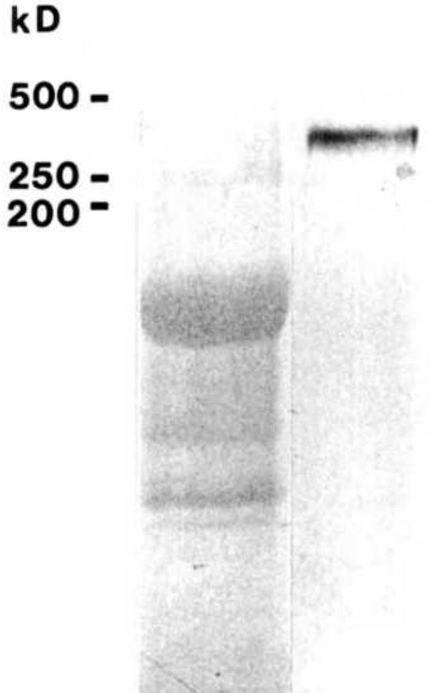

Figure 1. Western blot of high molecular weight proteins from the culture medium of Saos-2 cells (human osteosarcoma cell line) stained with polyclonal rabbit anti-rat tenascin serum (lane b). Lane a was stained for protein with Ponceau S. Reduced (250 kDa) and unreduced (500 kDa) human fibronectin and myosin (200 kDa) were used as molecular weight standards.

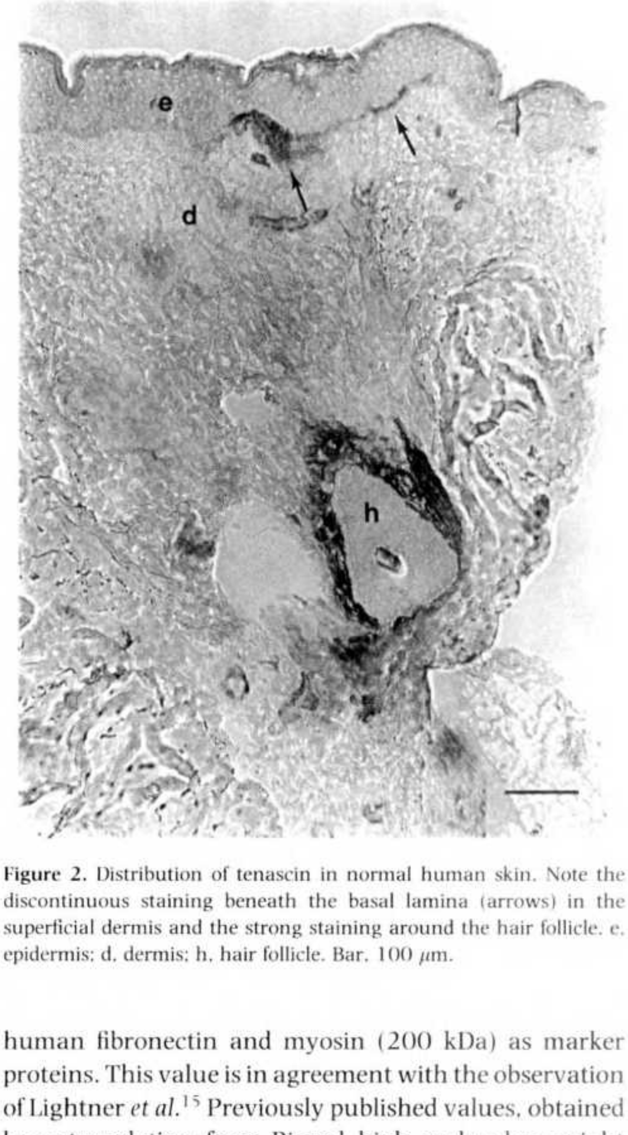

Figure 2. Distribution of tenascin in normal human skin. Note the discontinuous staining beneath the basal lamina (arrows) in the superficial dermis and the strong staining around the hair follicle. e, epidermis; d, dermis; h, hair follicle. Bar, 100 μm.

For immunohistochemistry, fixation of the tissues in acetone was the preferred method since paraformaldehyde fixation destroyed most of the tenascin reactivity. Pretreatment with hyaluronidase did not enhance tenascin staining. In normal skin, either obtained from healthy volunteers or from the clinically normal skin of patients with basal cell carcinoma, tenascin was discontinuously distributed in the upper dermis adjacent to the basal lamina varying from a patchy (Fig. 2) to a more pronounced distribution (Fig. 3). No relationship between tenascin expression in the upper dermis and the age of the donor was noted. A marked, continuous staining for tenascin was found in the connective tissue adjacent to the basement membrane of subepidermal capillaries, eccrine sweat ducts and hair follicles (Fig. 2).

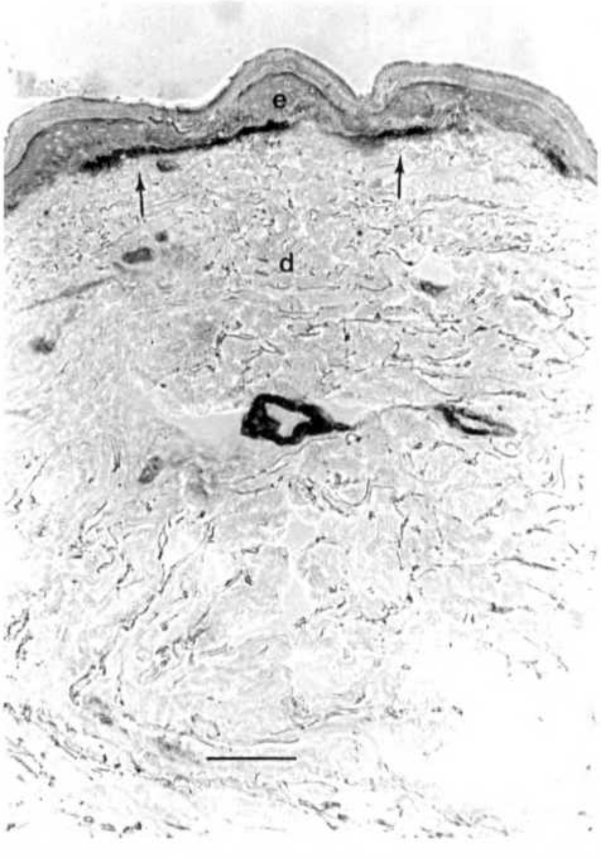

Figure 3. Distribution of tenascin (arrows) in normal human skin. Note the discontinuous staining in the upper dermis adjacent to the basal lamina. Tenascin is absent in the deep dermis except around blood vessels and adnexal structures. d, dermis; e, epidermis. Bar, 100 μm.

This is in agreement with previous findings on normal skin.[15] The distribution of tenascin is different from that of other extracellular matrix components such as fibronectin, laminin and heparan sulphate proteoglycan. The distribution of fibronectin was found to be diffusely distributed in the entire dermis but more pronounced in the upper dermis[15,21] (see also Fig. 4). This is in contrast with tenascin which is, in normal skin, focally distributed in the upper dermis and is absent in the deep dermis. The expression of laminin and heparan sulphate proteoglycan in normal skin was restricted to basal membranes (not shown).

As tenascin expression has been described in conditions of mesenchyme interacting with proliferating epithelium, we studied the presence of tenascin in psoriasis (a benign inflammatory disease with epidermal hyperproliferation), basal cell carcinoma (a non-metastasizing carcinoma), Bowen's disease (a precancerous dermatosis) and solar keratoses.

In the eight psoriatic patients studied a marked expression of tenascin was found compared to normal controls (Fig. 5). The dermal papillae were intensely stained and a continuous band of tenascin was found in the dermis along the basal membrane. The transition to dermal tissue negative for tenascin was rather abrupt, as shown in Figure 5. The expression of other extracellular matrix components differed from tenascin. As previously described,[21] fibronectin expression was slightly increased in lesional psoriatic skin compared to normal and uninvolved psoriatic skin. In contrast to tenascin which is sharply demarcated (Fig. 5), fibronectin was found diffusely throughout the dermis (Fig. 4). In serial sections stained for tenascin and fibronectin, it was found that the topography of tenascin expression did not match that of fibronectin. Apparently, expression of these extracellular matrix proteins is subject to distinct control mechanisms. The expression of laminin and heparan sulphate proteoglycan in psoriatic skin was, as in normal skin, restricted to basal membranes. In the clinically uninvolved skin of six out of eight psoriatic

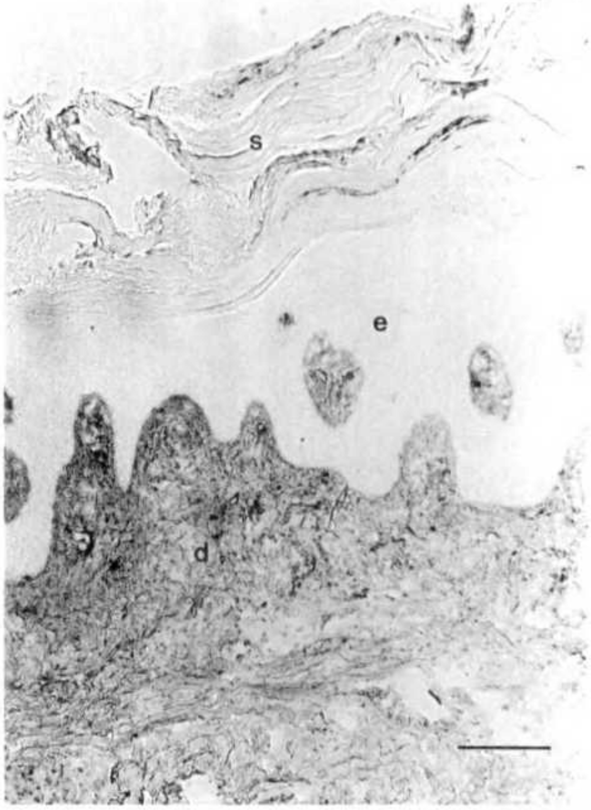

Figure 4. Psoriatic lesional skin stained for fibronectin. Fibronectin is diffusely present throughout the dermis. s, stratum corneum; e, epidermis; d, dermis. Bar, 100 μm.

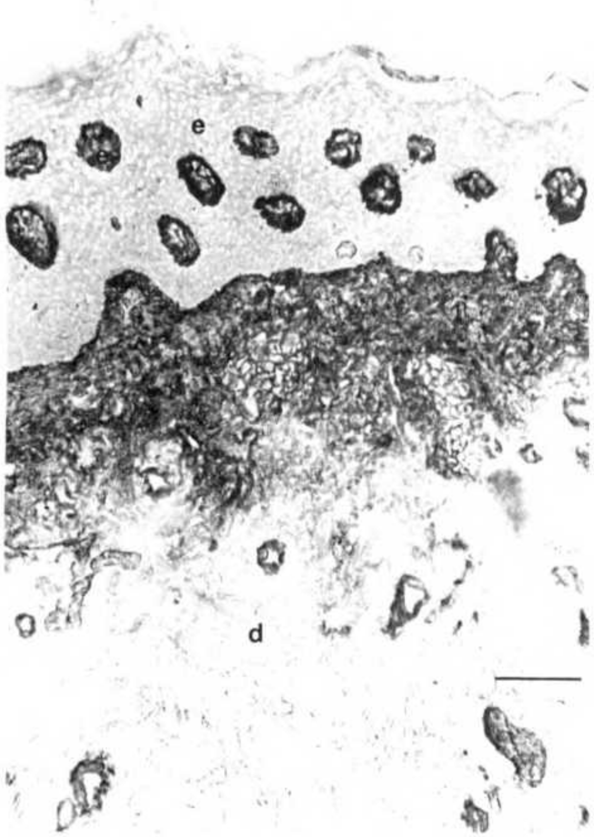

Figure 5. Distribution of tenascin in lesional psoriatic skin. Tenascin is present as a continuous band along the basal lamina and extends to the deeper dermis. Dermal papillae (in cross section) show strong staining. e, epidermis; d, dermis. Bar, 100 μm.

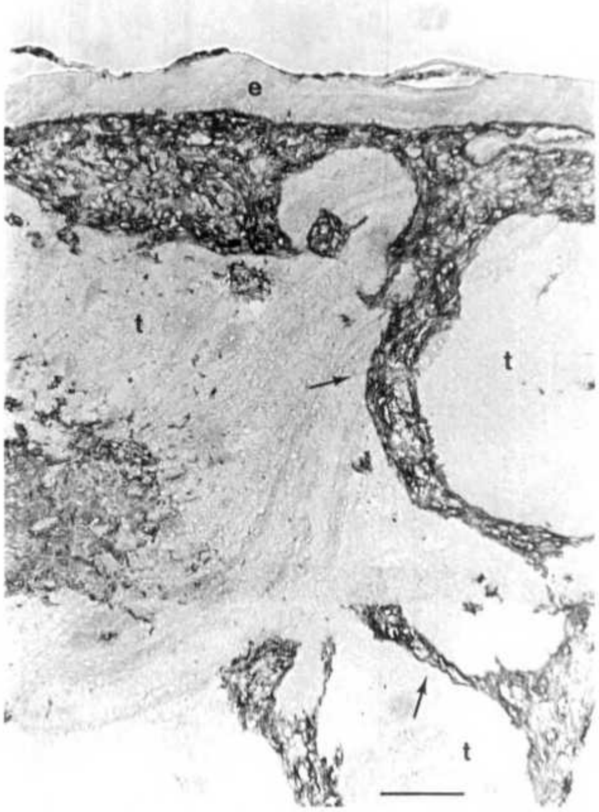

Figure 6. Basal cell carcinoma stained for tenascin. Both the subepidermal dermis and the tumour stroma are strongly positive. e, epidermis; t, tumour cells, arrows: tumour stroma. Bar, 100 μm.

patients, tenascin could be demonstrated as a more or less continuous band along the basal membrane. Whether the expression of tenascin in uninvolved psoriatic skin is increased compared to normal skin remains to be investigated by quantitative techniques.

In basal cell carcinomas the tumour stroma stained intensely for tenascin (Fig. 6). Fibronectin was also strongly expressed in the stroma of basal cell carcinomas as previously described.[22] However, there were slight differences in the distribution of both extracellular matrix proteins. Tenascin staining was most prominent directly adjacent to the basal membrane lining the tumour cell nests, whereas fibronectin was present more diffusely throughout the stroma (not shown). Staining for laminin and heparan sulphate was again limited to basal membranes (Fig. 7 and 8).

In Bowen's disease (Fig. 9) and in solar keratoses (Fig. 10) tenascin was strongly expressed in the dermal tissue adjacent to the keratinocyte clusters.

Discussion

We have investigated tenascin expression in normal human skin and in skin from diseases characterized by epidermal hyperplasia. As previously described for humans and rats, the normal skin showed a limited distribution of tenascin.[4,15] In hyperproliferative skin diseases, however, an abundant continuous expression of tenascin was found in the dermal compartment where tenascin expression is sparse and patchy in normal skin.

Tenascin is a recently discovered extracellular matrix protein which is probably identical to a number of other molecules described under various names.[5-9] In view of its ample expression during embryogenesis and its functioning as a surface adhesion molecule, it is likely to play a role as a morphogen.[23] Although the distribution in adults is limited,[24] tenascin can be re-expressed under pathological conditions as shown in breast tumors[1,9] and in wound healing.[4] Tenascin expression by fibro-

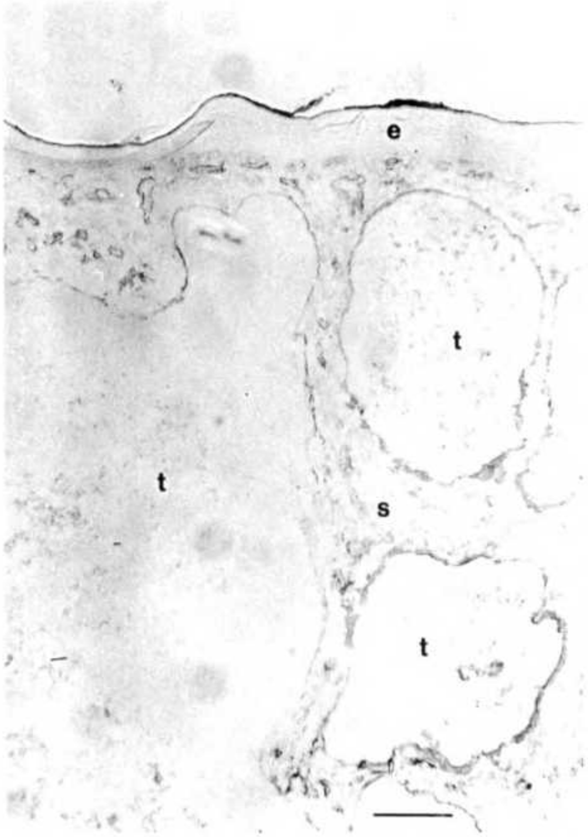

Figure 7. Distribution of laminin in basal cell carcinoma. Staining is mainly associated with basal membranes. e, epidermis; t, tumour cells; s, tumour stroma. Bar, 100 μm.

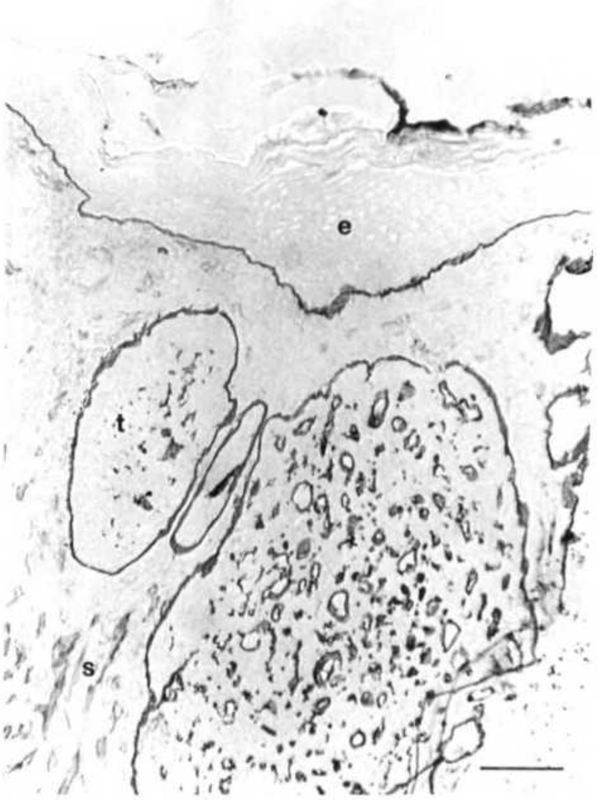

Figure 8. Distribution of heparan sulphate proteoglycan in basal cell carcinoma. Staining mainly associated with basal membranes. e, epidermis; t, tumour; s, tumour stroma. Bar, 100 μm.

blasts is in many instances directed by the activity of epithelial cells.[4,10] As epidermal cells readily migrate on a tenascin-containing substratum, induction of tenascin expression in wound healing could be seen as an attempt by the keratinocytes to close the wounded area.

At present few data are available on the expression of tenascin in human skin diseases. In view of the findings mentioned above we selected for this study a number of dermatoses in which keratinocyte proliferation is a prominent feature. In all cases a strong tenascin expression was found. Whether this phenomenon is directly related to epidermal activity remains to be established, since a weak to moderate expression of tenascin was also found in biopsies from a melanocytic naevus and cutaneous lupus erythematosus (not shown). Thus, tenascin expression is unlikely to be specific for a given disease or group of diseases. The observed correlation with malignancy as reported for breast tumours is not found in skin diseases, since both psoriasis, a benign disease, and a malignancy, basal cell carcinoma, showed similar strong expression.

Little is known about the function of tenascin in normal human skin and in the skin diseases described. In view of the proposed role of tenascin in wound healing, the reactions in psoriasis and skin carcinomas could be interpreted as a frustrated wound-healing process, in which the keratinocytes are continuously signalling the dermis to lay down a bed of tenascin. An interesting direction for future research is the possible role of tenascin in tumour metastasis. If the migration of epithelial cells is indeed favoured by a tenascin-containing substratum, it could be envisaged that tumours which induce the formation of tenascin in their stroma enhance metastasis. Obviously, expression of tenascin alone is not sufficient since basal cell carcinomas usually do not metastasize. It is, however, clear that interactions with stroma are important factors in malignancy.[25] We anticipate that further knowledge of the interaction of

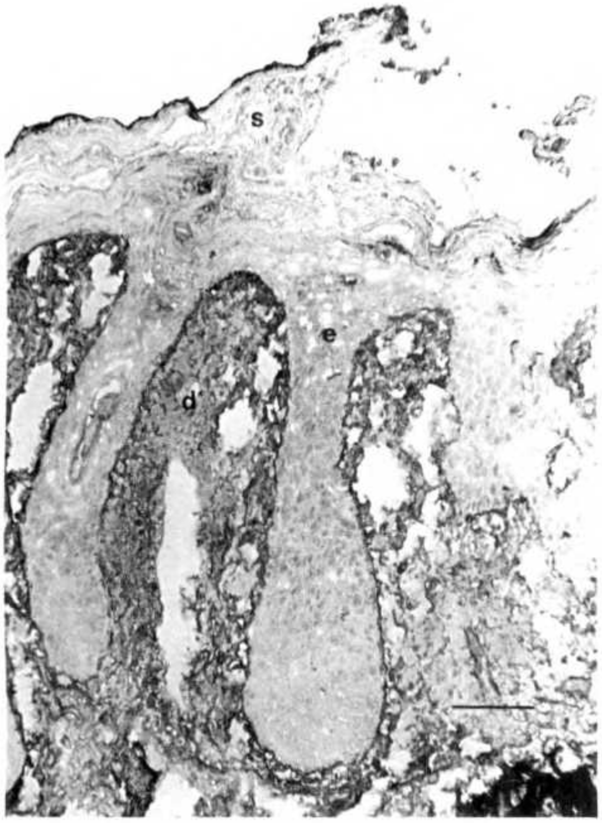

Figure 9. Tenascin staining in Bowen's disease. Strong staining in the elongated dermal papillae. e, epidermis; d, dermis; s, stratum corneum. Bar, 100 μm.

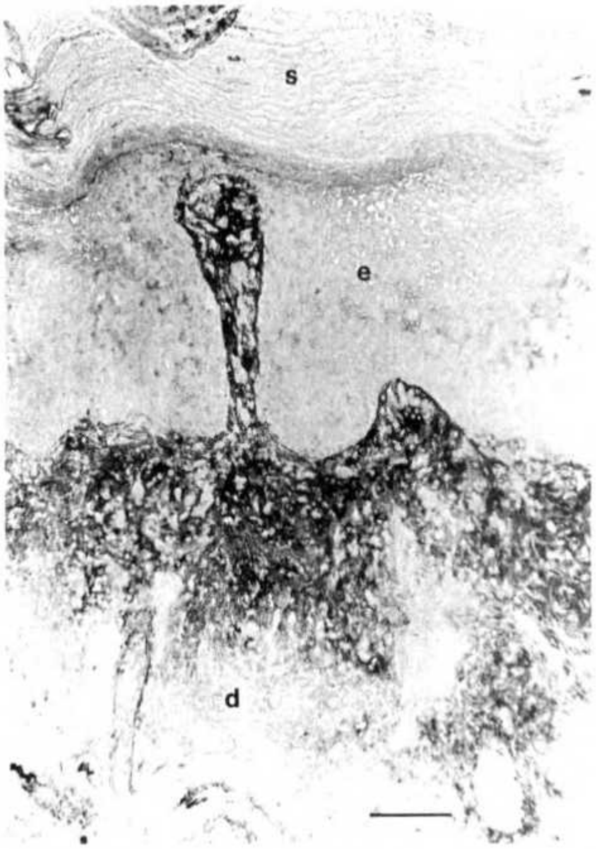

Figure 10. Tenascin staining in solar keratosis. Note the strong staining in the dermis beneath the dysplastic epidermal cells. e, epidermal cells; d, dermis; s, stratum corneum. Bar, 100 μm.

keratinocytes with dermal cells resulting in extracellular matrix modulation, will provide us with fundamental knowledge of the processes seen in wound healing and skin diseases.

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Physiology of the vellus hair follicle: hair growth and sebum excretion

U.BLUME,*† J.FERRACIN,* M.VERSCHOORE,*† J.M.CZERNIELEWSKI*† AND H.SCHAEFER*†

Centre International de Recherches Dermatologiques (CIRD), Valbonne, France
†Centre de Pharmacologie Clinique Appliquée à la Dermatologie (CPCAD), Hôpital Pasteur, Nice, France

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Summary The growth of vellus hair and the secretion of sebum from vellus hair follicles were measured on the forehead, cheek, chest, shoulder and back of healthy men and women aged 15–30 years. Hair growth was assessed by computerized image-analysis of photographs and sebum excretion by the use of Sebutape® followed by image analysis. The density of vellus hairs and the percentage of growing hairs were higher on the face than on the thorax (439 hairs/cm^2 with 49% growing hairs on the forehead compared with 85 hairs/cm^2 with 31·5% growing hairs on the back). The rate of growth ranged from 0·03 mm/day on the forehead to 0·13 mm/day on the back. The maximum length of vellus hair significantly decreased with age: otherwise hair growth was not affected by age or sex. Some variations in hair growth and sebum secretion were observed over a period of 3 months, but no consistent rhythms were detected. There was no obvious link between vellus hair growth and sebum excretion.

Vellus hair is uniformly spread over the body surface, except in regions of terminal hair growth and on the soles and palms.[1] Vellus hair grows postnatally and is fine, silky, generally unmedullated and unpigmented, rarely thicker than $\leq 30\ \mu$m and not longer than 2 cm.[2] It is produced by vellus hair follicles, which are small terminal hair follicles with a small follicle opening, disproportionately large sebaceous glands and a small hair bulb. Vellus hair follicles are considered to be non-androgen-depedent, but like terminal hair follicles undergo a growth cycle with cyclical changes of anagen, catagen and telogen periods.[3,4]

The activity of pilosebaceous units can be estimated by their products, the hair and the epidermal lipids.[5] Whereas hair growth dynamics depend on the rate of cell proliferation from the bulb matrix, sebum production involves both the kinetics of sebaceous cell turnover and the delivery of formed sebum to the skin surface.[6] Previous studies of the physiology and physiopathology of pilosebaceous units have focused mainly on terminal and sebaceous follicles and only a few have considered the physiology of the vellus hair follicle.

The purpose of the present work was to study the activity of the vellus hair follicle, to evaluate its hair growth cycle and sebum production, and to determine the factors influencing these processes. In addition, a potential correlation between hair growth and sebaceous gland activity was investigated in individual follicles.

Methods

Twenty healthy volunteers (10 male and 10 female), equally distributed into two age groups (15–20 and 25–30 years), were included in the study. Four areas presenting vellus hair were defined in each subject: the forehead, cheek, chest and back in females and, in males, the forehead, shoulder, chest and back. Only men with few or no terminal hairs on the chest were admitted. Female subjects were not under hormonal contraception and started the study within the first 3 days of their menstrual cycle.

The study was conducted within the facilities of CPCAD (Centre for Clinical Pharmacology Applied to Dermatology, Nice, France) from January to April 1988. The study protocol was approved by the local ethical committee and each subject signed an informed consent form prior to entry into the study.

Vellus hair growth

A macrophotographic record of the four precisely delimited areas (0·8 × 1·2 cm) was made for each individual

Correspondence: Dr U.Blume, Department of Dermatology, University Medical Center Steglitz, Free University of Berlin, Hindenburgdamm 30, D-1000 Berlin 45, Germany.

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at fixed time intervals of 7 days over a total period of 12 weeks.

Area determination. In order to keep the same precise test sites for the entire study, the stratum corneum was stained at each weekly visit with dansyl chloride[7,8] to form four fluorescent points on the corners of each delimited area; this staining was only visible under Wood light. A transparent plastic sheet, on which individualized anatomical sites were drawn (hair line, eyebrows, naevi, scars), was also used.

The phototrichogram. This is one of the most recently developed non-invasive methods for the evaluation of hair growth.[9–11] It can be used by combining macrophotography with computer-assisted image analysis[12] and allows one to observe hair growth activity and to distinguish growing from quiescent hairs. A photographic system, developed by L'Oreal (Paris, France) was used in the present study. This system is composed of an Olympus OM2 Spot/Program camera, a Zuiko 1:1 macro 80-mm objective with a telescopic auto tube 65-116, a close-up lens 80-mm macro and a T10 ring flash with a polarizing ring cross-filter (×1·7 magnification). A bar carrying a footplate at an angle of 90° with a glass slide window fixed under the body of the camera, is used to press vellus hair over its whole length to the skin surface, thereby providing a good photographic record.

Dye technique. Vellus hairs, generally colourless, were dyed with a brown oxidation dye (Crescendo,• L'Oreal, France) to ensure visualization on slides. Photographic follow-up of hair growth was made during the weekly visits. Vellus hairs on the trunk were cut short with scissors and facial vellus was shaved at 3-week intervals, starting on day 0. Coloured 10×15 cm photographs were reproduced (×8·9 magnification) from the slides obtained each week. These were evaluated visually and then submitted to image analysis.

Analysis of photographic documents. On a precisely delimited area of the photographs, each individual hair could be identified and followed over its growth cycle. The percentage of anagen hair, generally accepted as an index of hair follicle activity, was assessed by a separate count of growing and of non-growing hairs in the same area. In addition, individual follicle activity was observed.

Phototrichometry. Transparent sheets were superimposed on the photographs and the vellus hairs were precisely traced onto them before these transparencies were submitted to image analysis (Fig. 1). The Quantimet 520 programme (Quantimet 520, Cambridge Institute, U.K.) allowed the measurement of the lengths and number of hairs drawn on the transparencies. From this data, the density of vellus hairs per cm^2 and the mean growth rate of vellus hair per day could be established. The hair lengths were measured from day 0 to day 84. Weekly differences were evaluated and the mean growth rate of vellus hair was calculated. The mean maximum length of vellus hairs at the end of their growing stage was obtained from direct measurement of all quiescent hairs. The duration of the growing period was defined as starting with the emergence of an anagen hair and ending with the transformation into a quiescent hair and was calculated by division of the mean maximum length of a vellus hair by its growth rate per day.

Sebum excretion

The sebum absorbent tape, Sebutape• (Hermal Pharmaceutical Lab., Oak Hill, U.S.A.), allowed the measurement of sebum production from individual follicles.[13,14] The sebum pore patterns provided a permanent record of size and distribution of sebum droplets. For each evaluation of sebum excretion, Sebutape• was placed on the delimited, previously defatted area of the forehead and the chest. After a collection period of 60 min, it was carefully removed, placed on a sheet of black paper and photographed using a standardized photographic system. The Sebutape• slides were submitted to automatic image analysis (Quantimet 520, Cambridge Instruments) and the density of actively secreting follicles (number of excreting follicles per cm^2), the percentage of Sebutape• surface covered with sebum droplets (quantity of excreted sebum in 60 min) and the mean area of one droplet (quantity of sebum excreted by one follicle) were measured directly. In addition, the slides of sebum excretion record were compared with the records of hair growth.

Results

Results obtained for vellus hair growth and for sebum excretion were analysed separately for female and male volunteers and for the different anatomical sites (Table 1). All hair growth parameters revealed significant interzone differences between the face and body sites ($P < 0.01$).

An additional analysis was performed to determine the influence of age on vellus hair growth and sebum

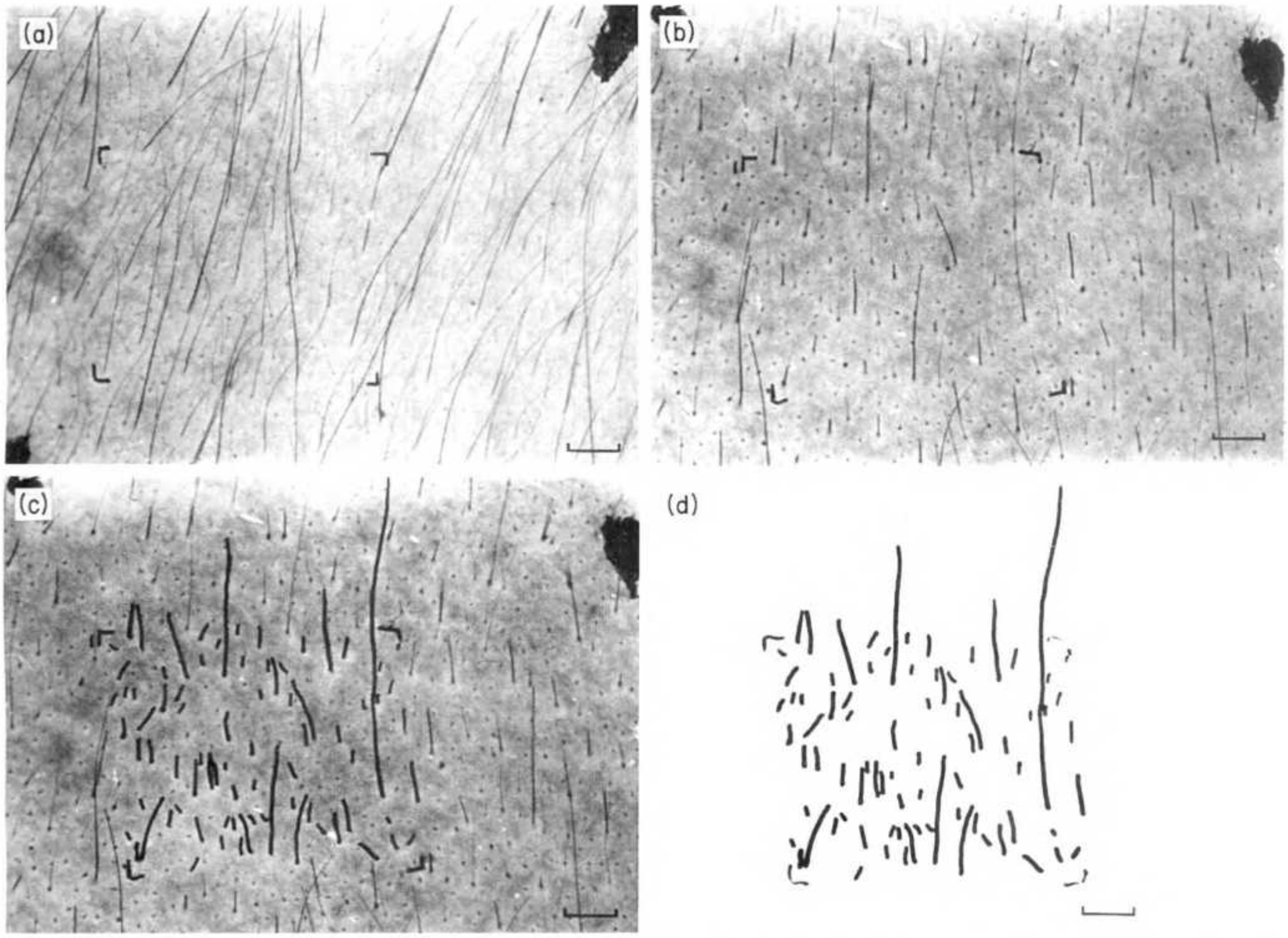

Figure 1. Phototrichometry of vellus hair on the cheek in a female subject (bar = 0·1 cm). (a) Vellus hairs before shaving at Day 0; (b) length of regrown vellus hair at Day 21; (c) length of grown vellus hair transcribed onto superimposed transparent sheet at Day 21; (d) transparent sheet of Day 21 submitted to image analysis.

Table 1. Vellus hair growth parameters in healthy subjects: females and males (mean ± SEM*)

	Density (hairs/cm^2)	Growth rate/day (mm/day)	Growing hair (%)	Duration of growing stage (days)
Females				
Forehead	448 ± 30·8	0·037 ± 0·005	48·5 ± 4·5	58·2 ± 7·2
Cheek	416 ± 37·7	0·060 ± 0·007	46·4 ± 4·8	78·6 ± 14·6
Chest	53 ± 1·4	0·109 ± 0·011	42·2 ± 2·4	40·8 ± 4·5
Back	93 ± 6·1	0·149 ± 0·012	30·9 ± 2·5	41·8 ± 1·8
Males				
Forehead	429 ± 37·5	0·025 ± 0·002	49·5 ± 4·8	63·4 ± 7·0
Shoulder	68 ± 5·5	0·119 ± 0·011	32·8 ± 2·2	51·2 ± 4·6
Chest	61 ± 5·0	0·107 ± 0·014	34·7 ± 2·9	50·7 ± 5·0
Back	77 ± 5·6	0·118 ± 0·014	32·0 ± 2·0	49·4 ± 2·9

* SEM, Standard error of the mean.

Table 2. Vellus hair growth parameters in healthy subjects (pooled sex: mean±SEM)

	Density of vellus hair (hairs/cm^2)	Growth rate/day (mm/day)	Growing vellus hair (%)	Maximum length (mm)	Duration of growing stage (days)
Forehead	439±23·7	0·031±0·003	49·0±3·0	2·0±0 3* 1·6±0·5†	60·8±4·9
Cheek	416±37·4	0·060±0 007	46·4±4·8	4·1±0 5* 3·4±0·4†	78·6±14·6
Shoulder	68+5 5	0·114±0·013	32·8±2·2	6·0±0·4* 4·3±0 4†	51·2±4·6
Chest	57±2·7	0·109±0·011	38·5±2·0	4·6±0·2* 3·9±0 4†	45·7±3·5
Back	85±4·5	0·134±0·010	31·5±1·6	6·2±0·4* 5·0±0·4†	45·6±1·9

* Age of volunteers: 15–20 years.
† Age of volunteers: 25–30 years.

excretion parameters. No age-dependent changes of vellus hair follicle activity were found when comparing the 15–20-year-old group (mean 17·3±0·8 years) to the 25–30-year-old group (mean 27·1±0·9 years) of volunteers, except for the mean maximum length of vellus hair, which decreased significantly with age.

After 12 weeks of continuous observation, the following data for vellus hair growth and sebum excretion were established.

Vellus hair growth

Density of vellus hair. Differences in the density of vellus hair on the forehead region (439 hairs/cm^2), cheek area (416 hairs/cm^2) and on the shoulder, chest and back (68, 57 and 85 hairs/cm^2, respectively) were noted (Table 2). The hair density of the areas common to both sexes (the forehead, the chest and the back) differed significantly ($P<0\cdot01$), showing the following rank order: forehead>back>chest.

Percentage of growing hairs. The interzone differences are presented in Table 2. The percentage of growing hair was significantly ($P<0\cdot01$) higher on the face, i.e. the forehead (49·0%) and the cheek (46·4%) than on the three observed regions of the trunk. The percentage of growing hair was significantly ($P<0\cdot01$) higher on the chest (38·5%) than on the shoulder (32·8%) or the back (31·5%). There were intra-individual variations in the number of growing hairs but they did not show any cyclical activity.

Growth rate of vellus hair/day. The average growth rate of vellus hair was significantly lower on the different regions of the face than on the thorax (Table 1). The mean growth rate was 0·03 mm/day on the forehead and 0·06 mm/day on the cheek. On the shoulder, chest and back, the hair growth rate was between 0·11 and 0·13 mm/day (Table 2). The intra-individual variation for this parameter was very low. Over the 3-month observation period, after cutting vellus hair every 3 weeks, no significant increase or decrease of the mean growth rate could be detected.

Table 3. Length of vellus hair (mm) in females and males of different age groups (mean±SEM)

	Females (15–20 years)	Females (25–30 years)	Males (15–20 years)	Males (25–30 years)
Forehead	2 2±0·2	1·6±0·9	1 7±0·3	1·5±0·1
Cheek	4·1±0·5	3·4±0·4	ND	ND
Shoulder	ND	ND	6·0±0·4	4·3±0·4
Chest	4·1±0·1	3·4±0·2	5·1±0·2	4·4±0·5
Back	5·9±0·3	4·9±0·4	6·4±0·5	5·0±0 4

ND, not done.

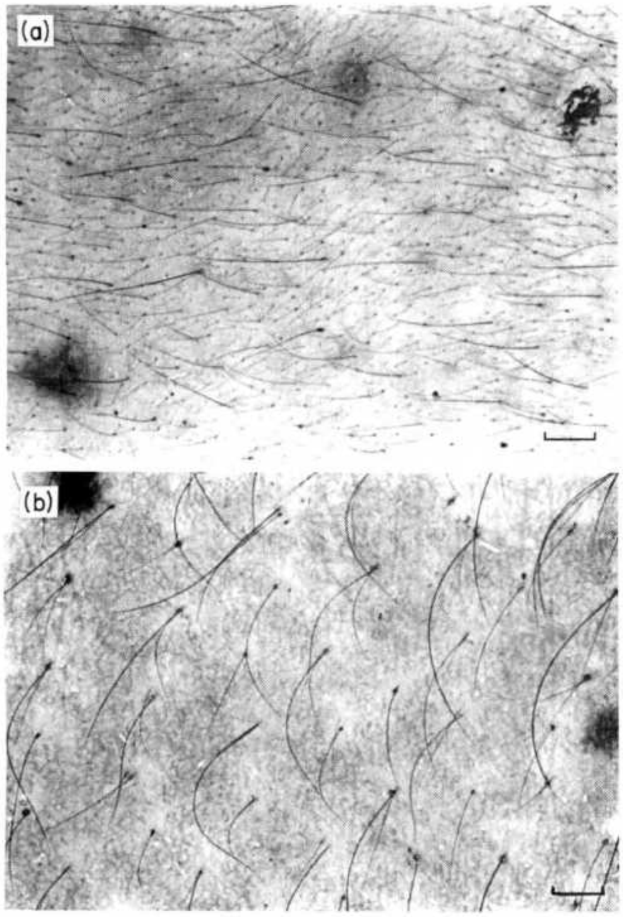

Figure 2. Pattern of vellus hair follicles in a female subject (bar = 0·1 cm). (a) on the forehead; (b) on the chest.

Maximum length of vellus hair. The mean maximum length of vellus hair on the regions common to both sexes (Table 3) was lower on the forehead (1·5–2·2 mm) than on the chest (3·4–5·1 mm) or the back (4·9–6·4 mm). In female subjects, cheek hairs were as long as those on the chest. In all test zones, except for the forehead, vellus hairs were longer in men than in women and decreased significantly ($P<0·01$) with age (Table 3).

Duration of the growing stage. The mean duration of the growing stage of vellus hair on the face lasted 35–100 days (forehead mean duration, 60 days; cheek, 80 days). On the thoracic areas the growing period lasted on average 45–51 days (Table 1).

Follicle pattern. It was noted that vellus hair on the forehead and cheek grew individually, whereas vellus hairs on the thorax were gathered in groups of three or four hairs. In all areas, interfollicular distances were relatively constant (Fig. 2). The diameter of the pilosebaceous duct orifices was smaller on the face than on the trunk.

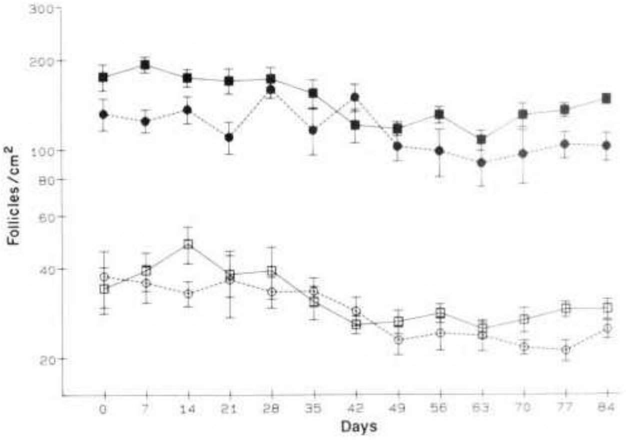

Figure 3. Density of actively secreting follicles: in females on the forehead (—■—) and on the chest (—□—), in males on the forehead (—●—) and on the chest (- -○- -), within 12 weeks of observation (log. scale, mean ± SEM).

Sebum excretion

Sebum excretion was measured on the forehead and on the chest. All subjects presented clinically normal sebum excretion. Sebum excretion parameters—density of actively secreting follicles and percentage of Sebutape® surface covered with sebum—were significantly higher on the forehead than on the chest ($P<0·01$) in both sexes (Figs 3 and 4).

For both sites, the mean density of actively secreting

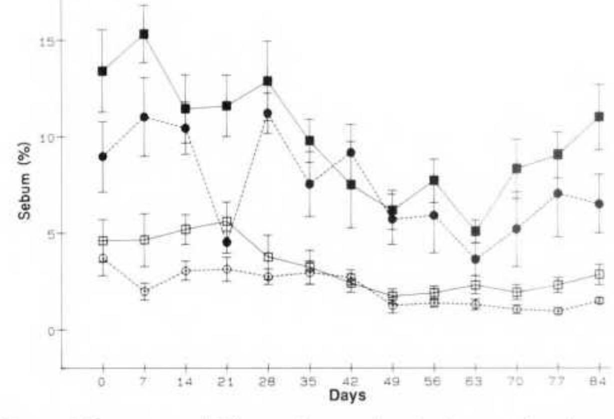

Figure 4. Percentage of Sebutape® covered with sebum: in females on the forehead (—■—) and on the chest (—□—), in males on the forehead (- -●- -) and on the chest (- -○- -), within 12 weeks of observation (mean ± SEM).

Table 4. Sebum excretion parameters measured by Sebutape® technique and computerized image analysis (mean±SEM)

	Number of actively secreting follicles (foll/cm^2)	% of Sebutape® covered with sebum (%)	Area of one droplet (mm^2)
Females			
Forehead	123·8±3·5	7·2±0·45	0·057±0·003
Chest	27·2±0·8	2·1±0·15	0·075±0·005
Males			
Forehead	98·5±5·5	5·5±0·64	0·050±0·003
Chest	23·1±0·8	1·3±0·09	0·054±0·003

follicles was higher ($P<0{\cdot}01$) in females (i.e. forehead 123·8 foll/cm^2) than in men (i.e. 98·5 foll/cm^2) (Table 4). The percentage of excreted sebum found on forehead and chest was also significantly higher ($P<0{\cdot}05$) in the female (i.e. forehead 7·2%) than the male volunteers (5·5%). A positive correlation was found between the density of actively secreting follicles and the percentage of Sebutape® surface covered with sebum on both test sites (forehead $r=0{\cdot}6$, chest $r=0{\cdot}7$).

The mean area of sebum droplets (i.e. the mean quantity of sebum excreted by one follicle) showed no significant difference between sexes or between the forehead (0·050–0·057 mm^2) and the back (0·054–0·075 mm^2) (Table 4). However, intra-individual and inter-individual heterogeneity in the size of the spots was evident. Intra-individual and inter-individual comparison of all sebum excretion parameters showed considerable variations within the 12-week study period, but these did not seem to follow any cyclical pattern (Figs 3 and 4).

Correlation between sebum excretion and vellus hair growth

A possible correlation between vellus hair growth and sebum production was evaluated by superimposition of Sebutape® images on vellus hair growth photographs. Sebum droplet area from quiescent hair follicles and the quantity of sebum excreted from growing hair follicles were compared. No correlation could be detected between these two functions of the pilosebaceous unit. These observations were confirmed by statistical tests based on canonical correlation analysis.

Discussion

In the present study, quantitative parameters of vellus hair growth and of sebum excretion from individual pilosebaceous units were evaluated in healthy volunteers. Vellus hair follicles showed significantly different activity depending on the anatomical location of the follicle, with higher values on the face than on the trunk, except for the mean growth rate and the mean maximum length of vellus hair. Thus, the function of vellus hair follicles varied with the body region in the same way as in terminal hair follicles.

The density of vellus hairs proved to be significantly higher on the forehead (439 hairs/cm^2) and cheek (416 hairs/cm^2) than on the trunk (57–85 hairs/cm^2). These results agree with earlier results reported by Szabo,[15] even though in his study the number of follicles on the face was higher than the present results. Our data are based on non-invasive techniques, where only hair follicles producing vellus hair reaching the skin surface were taken into consideration, whereas Szabo evaluated the number of hair follicles in histological sections.

The vellus hair follicle has been described as a rather inactive pilosebaceous unit, being more often in the resting than in the growing period (90%).[16,17] However, we found the percentage of growing vellus hair to be about 49% on the forehead and 46·4% on the cheek. Even the percentage of growing hair on the thorax which gave significantly lower results than on the face, was between 31·5–38·5%.

We also noted that the linear growth rate of vellus hair varied depending on its location on the body site. Vellus hairs on the forehead had the lowest growth rate (0·03 mm/day) and achieved a shorter mean maximum length (1·5–2·2 mm) than vellus hair on the other regions of the body (3·4–6·4 mm). However, vellus hairs on the second facial region, the cheek, grew faster (0·06 mm/day) than on the forehead and achieved in female subjects the same mean maximum length as vellus hair on the chest (3·4–4·1 mm). Hair growth rates and mean maximum length on the shoulder, chest and back were similar (0·109–0·134 mm/day and 4·3–6·4 mm).

Our results for vellus hair growth on the trunk are close to data for the growth rate of eyebrows, which grow at 0·16 mm/day,[18] whereas all other androgen-dependent hairs, like hairs of the scalp, chin and axilla, always grow at the rate of 0·20–0·35 mm/day.[19] Vellus hairs showed a 4–10-fold slower growth rate than terminal hair, but they reached a shorter mean maximum length than coarse terminal hairs.

A second important function of the vellus hair follicle, the production of epidermal lipids, was significantly higher on the face than on the thorax. Our 12-week follow-up of the sebum secretion of vellus hair follicles showed that in our population the density of actively secreting follicles and the quantity of excreted sebum was higher in females than in males. These findings contrast with previously published data.[20–23] In general, marked age and sex variations in sebum excretion rates can be observed after the age of 15 years with maximum values in the age group 15–40 years, but they are characterized by higher sebum excretion rates in males than in females.

Our population consisted of 10 female and 10 male volunteers aged 15–30 years, with a mean age of 22·2±0·9 years. In addition, the present data were obtained by a semiquantitative technique, the Sebutape®, whose primary use is the determination of the density of secreting follicles and the follow-up of individual pilosebaceous units, and not the precise quantification of the sebum secretion rate. Therefore, we do not claim that these results are generally representative and further investigations are needed for confirmation.

The number of actively secreting follicles and the quantity of excreted sebum showed considerable heterogeneity, but both parameters were nonetheless positively correlated. Similar heterogeneity in sebum excretion of individual follicles has previously been described by Piérard.[24,25] In his studies, Sebutape® measurements of the number of secreting follicles and the size of the sebum spots failed to demonstrate any cylic patterns. Neither did we detect any cylic variations of sebaceous gland activity, but there was a progressive fall of the number of sebum-secreting follicles and the percentage of secreted sebum within the 3-month study period, with a slight increase at the end of the observation period. As the study lasted from January to April, this decrease may have been a seasonal effect on sebaceous gland activity, with higher excretion rates and a higher number of secreting follicles in the winter.

Finally, even taking into account age, sex and location of the test site did not demonstrate any synchronization of vellus hair growth activity with sebaceous gland activity.

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Indomethacin inhibits the chemical carcinogen benzo(a)pyrene but not dimethylbenz(a)anthracene from altering Langerhans cell distribution and morphology

FIONA J.ANDREWS, G.M.HALLIDAY, C.K.NARKOWICZ* AND H.K.MULLER

*Departments of Pathology and *Medicine, University of Tasmania, Hobart, Tasmania, 7000, Australia*

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Summary Treatment of murine skin with the polyaromatic hydrocarbon carcinogens benzo(a)pyrene (BP) or dimethylbenz(a)anthracene (DMBA) for 3 weeks resulted in an increase and a decrease in epidermal Langerhans cell (LC) numbers, respectively, compared with solvent-treated skin. Implantation of subcutaneous indomethacin pellets prior to carcinogen treatment prevented the changes in LC numbers and morphology in BP, but not DMBA-treated skin. Indomethacin treatment was also found to reduce elevated prostaglandin E_2 $(PGE)_2$ levels in the skin of BP-treated mice, whereas PGE_2 levels were not significantly raised in DMBA-treated mice. There thus appears to be a link between altered prostaglandin levels and LC numbers in murine skin treated with BP, but not DMBA. In the latter, LC numbers were reduced by mechanisms not reversed by indomethacin. It is concluded that increased prostaglandin levels may contribute to the impairment of cutaneous immunity previously observed in BP-treated mice by altering LC density and morphology within the epidermis.

The distribution of epidermal antigen-presenting cells has been the subject of increasing interest over recent years, particularly in the light of reports documenting reduced Langerhans cell (LC) numbers in conditions predisposing to neoplasia such as cervical papilloma virus infections[1] and the oral mucosa of patients with AIDS.[2] Animal experiments have demonstrated depressed skin immune responses in mice with LC numbers reduced by ultraviolet irradiation,[3] or carcinogen treatment.[4] This has led to the suggestion that a reduction in epidermal antigen-presenting cells may allow neoplastic cells to evade the immune system enabling tumours to develop more readily.[5] The mechanisms involved in LC reduction in such models have not been fully elucidated. It is clear, however, that LC populations are not static, as electron microscopy studies have shown LC can migrate from the skin to local lymph nodes, presumably to present antigen to T lymphocytes.[6]

Initial studies from our laboratory demonstrated that treatment of the murine epidermis with the carcinogenic polycyclic aromatic hydrocarbon 7,12-dimethylbenz(a)anthracene (DMBA), depleted the LC population by more than 50%,[5] resulting in tolerance when sensitizing agents such as 2,4-dinitrofluorobenzene were applied to the LC-deficient site.[4] Specific, long-lived suppressor cells were demonstrated in these mice which could inhibit both the contact sensitivity response of immune mice[4] and the induction of contact sensitivity and humoral responses in naive mice.[7] Subsequently we have found that a population of DMBA-resistant, Ia positive, Thy-1-negative, I-J-restricted epidermal antigen-presenting cells are responsible for the induction of T suppressor cells in the local lymph nodes.[8]

Paradoxically we have also demonstrated that immune unresponsiveness to 2,4-dinitrofluorobenzene can arise in mice with increased numbers of LC. This occurred after 4 weeks of cutaneous application of the carcinogenic polycyclic aromatic hydrocarbon benzo(a)-pyrene (BP).[9] LC numbers remained raised during 24 weeks of treatment with BP and squamous cell carcinomas were observed in 42% of mice at the end of this period. It is unclear how unresponsiveness to 2,4 dinitrofluorobenzene arises in BP-treated mice with raised numbers of LC or how the LC numbers increase initially.

Experiments have shown that immune unresponsiveness to cutaneous antigen occurs in ultraviolet-irradiated mice even after LC numbers have returned to normal or are increased.[10] The continued tolerance to antigen in the presence of normal or increased LC could be reversed by pretreatment with the prostaglandin

Correspondence: Dr F J Andrews, Department of Surgery, Monash University Medical School, Prahran, Victoria, 3181, Australia.

synthetase inhibitor indomethacin, suggesting that increased prostaglandin levels may be responsible for the impaired immune response observed in these mice.[10] Studies have demonstrated that prostaglandin levels are increased after ultraviolet irradiation of the skin[11] or treatment of the epithelial-like cell line MDCK with BP.[12]

Prostaglandins (PG) are also thought to play a role in epidermal cell proliferation induced by tumour promotors.[13] Arachidonic acid, the precursor for prostaglandins, has been shown to alter numbers of LC when topically applied to normal murine skin[14] and cutaneous application of PGE_2 results in impaired contact sensitivity responses.[15] The aim of this study was to further investigate the role of prostaglandins in the control of LC numbers in the skin during chemical carcinogenesis. We compared the polyaromatic hydrocarbons BP and DMBA because previous studies have shown that these carcinogens have opposing effects on LC density.[9]

Methods

Animals

Male BALB/c mice 6–8 weeks of age were obtained from the University of Tasmania and used with the approval of the Ethics Committee. Animals were used in groups of six.

Indomethacin treatment

Controlled-release indomethacin pellets (Innovative Research of America, Toledo, OH, U.S.A.) containing 0·05 mg indomethacin in a biodegradable carrier, designed to release the indomethacin over a 3-week period (delivering 2·4 μg indomethacin/day) were implanted subcutaneously. Previous studies have shown that a daily dose of 1·25–2·5 μg of indomethacin from such pellets blocks skin prostaglandin synthesis.[16] Placebo pellets, containing the biodegradable carrier alone, served as controls.

Mice were anaesthetized by intraperitoneal injection of 0·036 mg/g sodium pentabarbitone (Nembutal; Biomac Labs, Asquith, NSW, Australia). A small incision (approximately 5 mm) was made in the shaved dorsal trunk skin at the base of the neck and the indomethacin or placebo pellet inserted distally, approximately 2 cm from the incision. The incision was sutured, swabbed with alcohol and the mice left to recover for 48 h prior to treatment with carcinogen. All incisions remained free from infection and mice receiving indomethacin pellets did not suffer from gastrointestinal bleeding.

Carcinogen treatment

Benzo(a)pyrene (BP 0·5% in acetone; Serva, Heidelberg) was applied twice weekly to the shaved dorsal trunk skin in 20 μl volumes. Control mice received acetone alone. 7,12-dimethylbenz(a)anthracene (DMBA 1%; Sigma, U.S.A.) was suspended in equal volumes of lanolin and liquid paraffin and applied to the shaved dorsal trunk once a week. Control mice received the paraffin/lanolin alone. Carcinogens and solvents were applied directly above the implanted pellets and treatments were continued for a total of 3 weeks.

Preparation of epidermal sheets

Mice were killed by cervical dislocation and the dorsal skin shaved and depilated using a thioglycallate preparation (Veet; Reckitt and Colman, U.K.). Skin samples (2 × 2 cm) were dissected from the murine dorsal trunk skin so that the pellet was located on the undersurface of the top edge of the skin, 1 cm from either side. The pellet was then teased away from the skin undersurface and the skin samples bisected down the vertical axis to form two identical sheets, which were adhered to cellophane tape. Skin sheets were then incubated in phosphate-buffered saline (PBS) containing 20 mM tetrasodium ethylene-diaminetetra-acetic acid at pH 7·4 for 2 h at 37°C. After incubation the epidermis was separated from the dermis with the aid of fine forceps and a dissecting microscope.

Identification of LC in epidermal sheets

LC were identified by staining for MHC class II antigen (Ia) or beta-glucuronidase (BG) activity as previously reported.[9] For demonstration of LC Ia antigens, the left side of the epidermal skin sample from each mouse was first fixed in acetone for 20 min at room temperature. The sheets were then washed in PBS followed by overnight incubation with the mouse monoclonal anti-Ia^d (MK-D6)[17] at 4°C. Sheets were then washed with PBS and incubated with peroxidase conjugated rabbit anti-mouse immunoglobulin (Dakopatts, Denmark), diluted in PBS, for 2 h at room temperature. Sheets were then washed again in PBS. Ia positive cells were demonstrated by staining with 0·5 mg/ml diaminobenzidine hydrochloride (Sigma, U.S.A.) in PBS containing 0·02% hydrogen peroxide, for 10 min. Washed specimens were mounted in 10% glycerol in PBS for microscopic examination.

The histochemical identification of BG activity in LC was carried out using the method of Hayashi *et al.*[18] Briefly, the right side of the epidermal sample from each mouse was fixed in 2% cacodylate-buffered formalin (pH 7·3) for 30 min followed by washing in PBS. Sheets were then incubated in 0·2 M sodium acetate/acetic acid buffer containing 10% gelatin solution (5% gelatin in sterile water; May & Baker Chemicals, Essex, U.K.), 1·8 mM pararosanilin hexazonium and 0·25 mM Naphthol AS-BI glucuronic acid (Sigma, U.S.A.) in 0·1 M $NaHCO_3$ for 4 h at 37°C with gentle agitation. After washing in PBS, sheets were counterstained in 0·1% methyl green, washed and mounted in PBS glycerol as before.

Enumeration of LC

LC were visualized by light microscopy and counted in two locations; directly above the area of the pellet (area A) and approximately 2 cm from the area of the pellet (area B) in both Ia- and BG-stained sheets. Three fields (0·2891 mm^2) were examined in each of the two areas, thus LC were counted in a total of 12 fields from each mouse.

The percentage of LC demonstrating a dendritic morphology (i.e. > three dendrites/cell) in cell counts from Ia-stained sheets were also recorded.

Measurement of PGE_2 in mouse skin

Following 3 weeks of carcinogen treatment, groups of mice were killed by cervical dislocation and the dorsal skin shaved. Extraction of PGE_2 from murine skin was carried out using a modification of the method of Ujihara *et al.*[19] Squares of skin 2 × 2 cm were removed down to the level of the muscle and homogenized for 1 min in a pestle and mortar precooled in liquid nitrogen. The homogenized skin was quickly transferred to a preweighed plastic vial containing 0·5 ml analytical grade methanol to prevent further prostaglandin synthesis and reweighed. Following the addition of 4·5 ml acidified water (pH 3, citric acid), samples were sonicated on ice for 10 s using a MSE sonicator and then centrifuged for 10 minutes at 1000 *g* at 4°C. Skin supernatants were then collected and stored at −70°C.

Bond Elut C^{18} extraction columns (Analytichem International, Harbour City, CA, U.S.A.) were prepared by washing successively with 2 ml each of methanol and acidified water (pH 3, citric acid). Mouse skin extracts were then applied to the columns with negative pressure and washed sequentially with 2 ml each of water (pH 3, citric acid), methanol/water (10:90) and benzene. Bond Elut Si columns were prepared with 5 ml benzene/ethylacetate (80:20). The organic extracts were eluted from the C^{18} columns on to the Si columns with 1 ml ethylacetate. The Si columns were then treated sequentially with 1 ml each of benzene/ethylacetate (60:40), benzene/ethylacetate/methanol (60:40:2) and benzene ethylacetate/methanol (60:40:10). PGE_2 was eluted with 5 ml benzene/ethylacetate/methanol (60:40:30). Solvent was removed under N_2.

Analysis of PGE_2 content in samples was performed using reagents and methods provided in a Du Pont PGE_2 ^{125}I radioimmunoassay kit. Sample extracts were reconstituted in 1 ml assay buffer and passed through 0·22 μm filters (Millex, Millipore Products Division, Bedford, MA, U.S.A.) prior to radioimmunoassay. A standard curve was prepared by diluting the reconstituted PGE_2 provided in the kit. Sample radioactivity was counted using a Rackbeta 1219 scintillation counter and PGE_2 content calculated from the standard curve.

Statistical analysis

Comparison of data was carried out by analysis of variance using a Least Significant Difference Test. A *P* value of less than 0·01 was regarded as significant.

Results

Indomethacin did not affect the numbers or morphology of LC in the control, solvent-treated mice as there was no significant difference between numbers or morphology of LC in these groups. There was also no significant difference between LC numbers and morphology in area A (directly above the implanted pellets) and area B (approximately 2 cm from the pellets) within any of the particular groups studied, suggesting that indomethacin in this form, is able to exert its actions for up to 2 cm from the site of implantation.

Effects of BP on LC numbers

Treatment of the murine skin with BP for 3 weeks resulted in a significant increase in Ia- and BG-stained LC compared with LC from control acetone-treated mice (Table 1, Figs 1a and b).

Effects of indomethacin on LC numbers in BP-treated skin

Indomethacin treatment resulted in a significant reduction in Ia- and BG-stained LC in BP-treated mice compared with LC from BP-treated mice with placebo pellets (Table 1, Fig. 1c). The increase in LC numbers in

Table 1. LC/mm^2 in epidermal sheets from control (acetone-treated) and BP-treated mice with placebo (plac) or indomethacin (indo) subcutaneous implants. Samples taken from two areas, A and B and the LC identified by using two markers, MHC class II antigen (Ia) or beta-glucuronidase (BG)

	Mean LC/mm^2 (±SD)			
	Ia		BG	
Treatment	A§	B§	A§	B§
Acetone/plac	894±83*	869±105*	914±92*	947±109*
Acetone/indo	892±50†	920±68†	794±82†	819±58†
BP/plac	1387±207*‡	1309±174*‡	1272±173*‡	1221±122*‡
BP/indo	842±115†‡	807±96†‡	907±184†‡	788±42†‡

*Acetone/plac (area A and B, Ia and BG) vs. BP/plac (area A and B, Ia and BG) $P<0{\cdot}001$.
†Acetone/indo (area A and B, Ia and BG) vs. BP/indo (area A and B, Ia and BG) NS.
‡BP/plac (area A and B, Ia and BG) vs. BP/indo (area A and B, Ia and BG) $P<0\ 001$.
§Area A, directly above implant; area B, 2 cm from implant.

BP-treated mice was reduced by indomethacin such that there was no significant difference between LC numbers in indomethacin/BP, acetone/indomethacin or acetone/placebo treated mice using either staining technique (Table 1).

Effects of DMBA on LC number

Application of DMBA to the skin of mice with placebo pellets resulted in a significant decrease in Ia- and BG-stained LC compared with LC from control mice that received placebo pellets and treatment with paraffin lanolin (Table 2, Figs 1d and e).

Effects of indomethacin on LC numbers in DMBA-treated skin

Indomethacin treatment had no effect on the numbers of LC in DMBA-treated skin (Fig. 1f). There was no significant difference in LC numbers from the skin of DMBA/indomethacin-treated mice and DMBA/placebo-treated mice using either staining technique (Table 2). In addition, Ia- and BG-stained LC numbers from DMBA/indomethacin-treated mice remained significantly lower than those from paraffin lanolin/indomethacin-treated mice (Table 2).

Effects of indomethacin on LC morphology in carcinogen-treated mice.

BP treatment resulted in a significant reduction in the percentage of LC with dendritic morphology (i.e. >3 dendrites/cell) (Table 3). However, indomethacin was found to prevent the reduction in dendritic morphology in BP-treated skin.

DMBA treatment was also found to reduce significantly the percentage of LC with dendritic morphology in epidermal sheets (Table 4) but to a far greater extent than BP (Figs 1b and e). Indomethacin did not restore the percentage of LC with dendritic morphology in DMBA-treated epidermal sheets as there was no significant difference between the percentage of dendritic LC in DMBA/indomethacin- and DMBA/placebo-treated skin.

Effects of indomethacin on PGE$_2$ levels in BP- and DMBA-treated skin

BP treatment caused a significant increase in skin PGE$_2$ levels compared with levels in normal murine skin (Table 5). Indomethacin treatment significantly reduced PGE$_2$ levels in BP-treated skin. DMBA treatment also increased skin PGE$_2$ levels but the results were not significant. In addition, indomethacin did not reduce PGE$_2$ levels in DMBA-treated skin.

Discussion

The polyaromatic carcinogens BP and DMBA appear to have opposing effects on Langerhans cells (LC). The results of this study confirm our previous findings that cutaneous application of the carcinogen BP leads to an increase in numbers of epidermal LC and alterations in LC dendritic morphology.[9] Our present results suggest that the increase in LC density and alterations in LC morphology may be attributed to the direct or indirect effects of prostaglandin.

Studies have shown that PGD$_2$ and PGE$_2$ are the most abundant eicosanoids in normal skin samples[19] and are synthesized in preference to other prostaglandins when

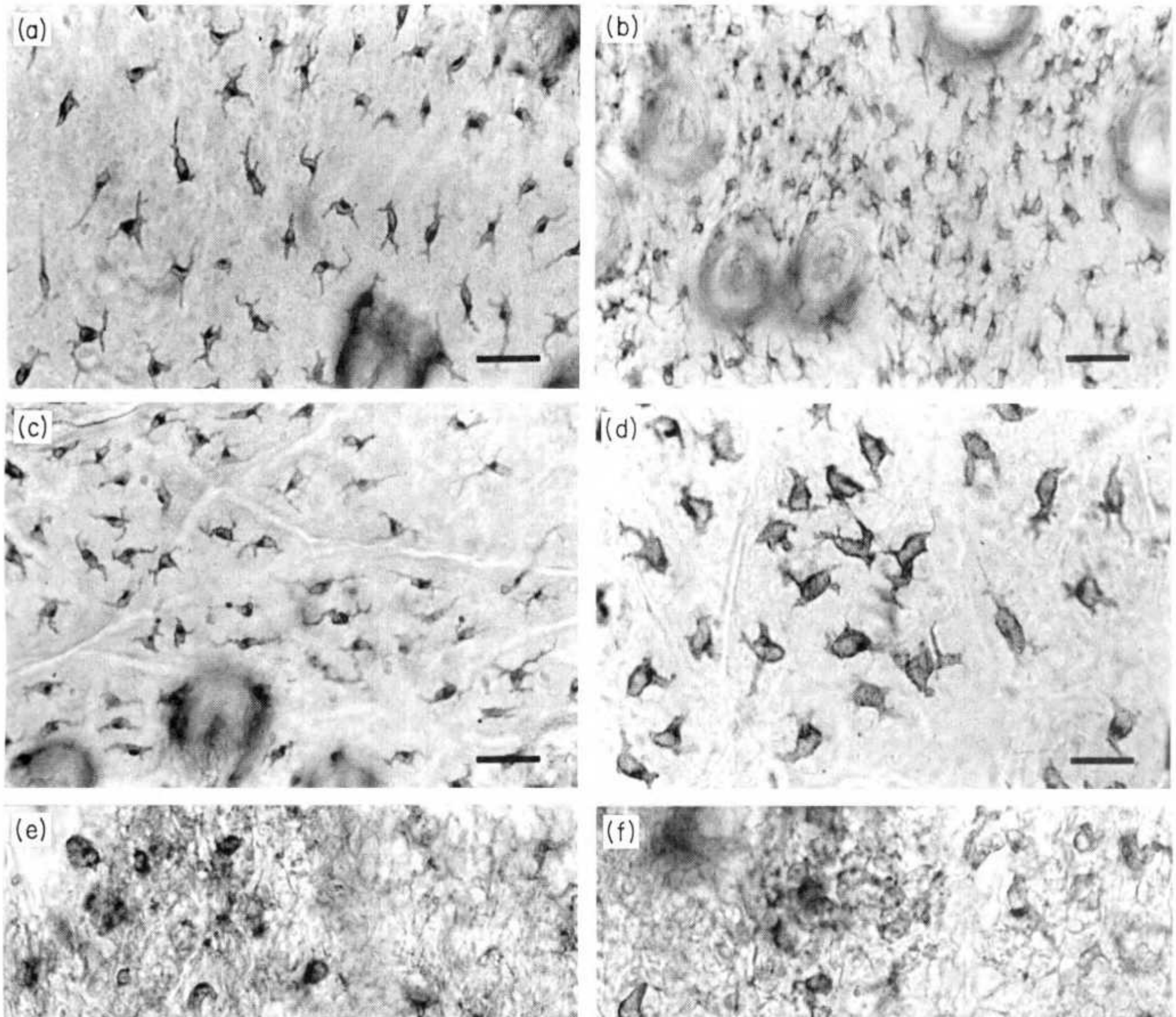

Figure 1. LC distribution and morphology in epidermal sheets stained for Ia from mice treated with (a) acetone/placebo; (b) BP/placebo; (c) BP/indomethacin. Bar = 16 μm; (d) paraffin lanolin/placebo; (e) DMBA/placebo; (f) DMBA/indomethacin. Bar = 8 μm.

intact skin[19] or epidermal cells[20] are exposed to excess arachidonic acid. For these reasons PGE_2 levels were measured in our experiments. In normal murine skin, PGE_2 levels were found to be low and in line with levels reported by Ujihara *et al.*[19] Inhibition of prostaglandin synthesis by indomethacin had no effect on numbers of LC demonstrated by staining for Ia antigens or BG activity in the normal murine skin. Topical application of BP for 3 weeks however, significantly increased the levels of PGE_2 and at the same time increased overall numbers of LC whilst reducing the percentage of LC with dendritic morphology. Indomethacin reduced the increase in LC numbers, the changes in LC morphology and the raised skin PGE_2 levels.

Table 2. LC/mm^2 in epidermal sheets from control (paraffin lanolin-treated) and DMBA-treated mice with placebo (plac) or indomethacin (indo), subcutaneous implants. Samples were taken from two areas, A and B and the LC identified by using two markers, MHC class II antigen (Ia) or beta-glucuronidase (BG)

	Mean LC/mm^2 (±SD)			
	Ia		BG	
Treatment	A§	B§	A§	B§
PL/plac	899±84*	836±47*	789±40*	729±90*
PL/indo	850±130†	809±94†	830±50†	778±60†
DMBA/plac	358±57*‡	301±69*‡	361±80*‡	352±103*‡
DMBA/indo	368±42†‡	342±61†‡	378±55†‡	363±29†‡

*PL/plac (area A and B, Ia and BG) vs DMBA/plac (area A and B, Ia and BG) $P<0{\cdot}001$.
†PL/indo (area A and B, Ia and BG) vs DMBA/indo (area A and B, Ia and BG) $P<0{\cdot}001$.
‡DMBA/plac (area A and B, Ia and BG) vs. DMBA/indo (area A and B, Ia and BG) NS.
§Area A, directly above pellet; area B, 2 cm from pellet.

Table 3. Percentage of LC with dendritic morphology in epidermal sheets from control (acetone-treated) and BP-treated mice with indomethacin (indo) or placebo (plac), subcutaneous implants

	Mean % dendritic LC (±SD)	
	Area§	
Treatment	A	B
Acetone/plac	96·5±2·4*	95·8±1·4*
Acetone/indo	98·7±0·8†	98·7±0·5†
BP/plac	89·8±1·5*‡	90·3±0·6*‡
BP/indo	96·5±2·4†‡	95·8±1·5†‡

*Acetone/plac (area A and B) vs. BP/plac (area A and B) $P<0{\cdot}001$
†Acetone/indo (area A and B) vs BP/indo (area A and B) NS
‡BP/plac (area A and B) vs BP/indo (area A and B) $P<0{\cdot}001$.
§Area A, directly above pellet; area B, 2 cm from pellet.

Table 4. Percentage of LC with dendritic morphology from epidermal sheets of control (paraffin lanolin-treated) and DMBA-treated mice with indomethacin (indo) or placebo (plac), subcutaneous implants

	Mean % dendritic LC (±SD)	
	Area§	
Treatment	A	B
PL/plac	91·5±3·4*	92·5±4·2*
PL/indo	96·2±1·5†	96·2±1·2†
DMBA/plac	63·3±4·1*‡	68·3±14·2*‡
DMBA/indo	67·0±19·6†‡	57·0±2·0†‡

*PL/plac (area A and B) vs. DMBA/plac (area A and B) $P<0{\cdot}001$
†PL/indo (area A and B) vs. DMBA/indo (area A and B) $P<0{\cdot}001$.
‡DMBA/plac (area A and B) vs. DMBA/indo (area A and B) NS
§Area A, directly above pellet; area B, 2 cm from pellet.

Table 5. Levels of PGE_2 in murine skin treated with BP or DMBA in the presence or absence of indomethacin (indo), subcutaneous implants

Treatment	Mean PGE_2 ng/g wet wt (±SD)
Control	1·85±1·36*‡
BP	13·53±1·59*†
BP/indo	2·49±0·85†
DMBA	7·62±7·48‡§
DMBA/indo	8·92±3·26§

*Control vs. BP treated $P<0{\cdot}001$
†BP vs. BP/indo $P<0{\cdot}001$.
‡Control vs DMBA NS.
§DMBA vs DMBA/indo NS.

There is evidence that Ia negative Langerhans cells can be induced to express Ia by cytokines leading to an increase in Ia positive LC within the epidermis.[21] However, we believe that BP induced a genuine increase in LC density, given that both Ia- and BG-stained LC increased in a similar manner in our study.

LC normally make up approximately 2–3% of the epidermal cell population. Factors which control LC density in the epidermis are unknown. However, LC populations are not static as LC can migrate from the skin to present antigen to T cells in the local lymph nodes.[6] The increase in numbers of LC in BP-treated mice may be a direct result of an increased migration of LC precursors into the skin. Recent reports have suggested, however, that LC are also capable of division within the epidermis.[22] Alternatively, BP treatment may lead to the retention of LC within the epidermis, either alone, or together with an increase in LC migration and division.

There is evidence that eicosanoid levels within the skin may effect LC numbers. Application of 0·05 g/dl arachidonic acid to the murine epidermis increased numbers of LC.[14] Higher doses of arachidonic acid (2 g/dl) decreased LC numbers but this was attributed to toxic detergent effects.[15] Application of 1 or 25 μg PGE_2/day for 1 week to murine ear skin had no effect on LC numbers.[15] It is possible therefore that the metabolism of arachidonic acid to prostaglandin is the stimulus for the increase in LC numbers rather than prostaglandin itself. It is also possible that leukotrienes may be responsible for the increase in LC observed in the former study.[14] However, this is unlikely based on the results of our study, since indomethacin treatment, which should not decrease levels of leukotrienes, reduced LC numbers to normal levels in BP-treated mice.

Increased levels of skin prostaglandins induced by BP treatment may be responsible for the impairment of contact sensitivity reactions observed in our earlier studies,[9] particularly as PGE_2 has been shown to inhibit contact sensitivity when applied directly to the murine skin.[15] We have previously suggested that BP treatment leads to cutaneous immunosuppression by inducing retention of LC within the skin[9] and it is possible that this is mediated by increased prostaglandin levels. The dendritic nature of LC may also be connected with antigen presentation and interaction of LC with T lymphocytes. BP treatment caused a small but significant reduction in the dendritic nature of LC. This too, appears to be linked to increased prostaglandin levels, given that indomethacin treatment significantly improved the dendritic nature of BP-treated LC.

Treatment of the murine epidermis with DMBA for 3 weeks gave different results to treatment with BP, with a reduction in LC numbers and gross changes to their dendritic morphology. LC numbers were reduced and their dendritic morphology altered after DMBA treatment in a prostaglandin-independent manner because indomethacin treatment did not reverse these effects. In addition, skin prostaglandin levels in DMBA-treated mice were not significantly increased above normal levels and were unaffected by indomethacin. The reduction in numbers of LC in DMBA treated mice may have been a consequence of cell death or an increase in LC migration from the epidermis. Results of recent studies support the latter hypothesis.[8]

We have shown that suppressor cells resulting from application of contact sensitizers to DMBA treated skin are activated in the local lymph nodes by a population of Ia positive, Thy-1-negative, 1-J-restricted epidermal antigen-presenting cells.[8] We have postulated that DMBA treatment disturbs the relative distribution of antigen-presenting cells which activate helper and suppressor lymphocytes. As a result, epidermal antigen-presenting cells which activate helper cells appear to leave the epidermis, allowing antigen-presenting cells which activate suppressor cells to predominate, resulting in tolerance rather than immunity.[8]

In conclusion we have demonstrated that indomethacin prevents the increase in LC numbers, changes in LC morphology and raised PGE_2 levels in mice treated with the polyaromatic hydrocarbon carcinogen BP, but has no effects on LC numbers in skin treated with a different polyaromatic hydrocarbon carcinogen, DMBA. We suggest that increased levels of prostaglandin might be involved in LC retention within the skin and failure to develop an immune response to cutaneous antigen in BP-treated mice. Studies are underway to investigate the antigen-presenting status of LC in murine skin pretreated with indomethacin and exposed to BP.

Acknowledgments

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Allergic contact and photocontact dermatitis due to psoralens in patients with psoriasis treated with topical PUVA

A.TAKASHIMA, K.YAMAMOTO, S.KIMURA, Y.TAKAKUWA AND N.MIZUNO
Department of Dermatology, Nagoya City University Medical School, Nagoya, Japan

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Summary The incidence and clinical features of allergic contact and/or photocontact dermatitis due to psoralens were examined in 371 patients with psoriasis treated with topical PUVA. The psoralen derivatives used in the study were 8-methoxypsoralen (8MOP), 3-carbethoxypsoralen (3CPs), 4,6,4′-trimethylangelicin (TMA) and 7-methyl pyridopsoralen (MPP). Of 371 patients treated with 8MOP, three (0·8%) developed an acute dermatitis in the PUVA-treated areas. This incidence was significantly lower ($P<0{\cdot}01$) than that for 3CPs (four of 10 patients) or that for TMA (six of 17 patients). None of the seven patients receiving MPP on PUVA had a reaction. It was confirmed that these dermatitis reactions were due to contact and/or photocontact allergy to psoralens by several methods that included patch and photopatch tests, photopatch test mapping, determination of the minimal erythema dose (MED) and immunohistochemistry.

Photochemotherapy with psoralens and long-wave ultraviolet light (UVA) has been widely used in the treatment of various skin disorders including psoriasis. One adverse effect of PUVA therapy is the development of severe dermatitis. Similar dermatitis reactions can be induced by contact with plants that contain psoralens following sun exposure as a phytophotodermatitis.[1] Because of the well-known phototoxic capacity of psoralens most of these dermatitis reactions have been considered to be phototoxic rather than photoallergic. Only a few cases of photoallergy to psoralens during PUVA therapy [2-4] or from plants[5] have been reported. So far no systematic study has been carried out to determine the incidence of allergic reactions to psoralens in patients on PUVA treatment

8-Methoxypsoralen (8MOP) is one of the most widely used psoralens in PUVA therapy and is known to promote DNA interstrand cross-linking following UVA irradiation by inducing two cyclo-C-4 additions between the 3,4 and 4′,5′ double bonds and the 5,6 double bond of the two pyrimidine bases.[6] To reduce the potential carcinogenic effect of PUVA, attributable in part to the above feature of psoralens,[7] several institutions have used monofunctional instead of bifunctional psoralens.[8,9] We have previously reported that 3-carbethoxypsoralen (3CPs), 4,6,4′-trimethylangelicin (TMA) and 7-methyl pyridopsoralen (MPP) are effective in the topical PUVA therapy of psoriasis.[10-12] This paper describes cases of photocontact allergy to 8MOP as well as to these monofunctional compounds and compares the antigenic potency of 8MOP and the monofunctional psoralens.

Methods

Patients

A total of 371 patients with severe psoriasis were treated with topical PUVA at Nagoya City University Hospital between 1971 and 1989. All patients received our standard topical PUVA regimen using 8MOP as a photosensitizer, and some of them were treated additionally with 3CPs, TMA or MPP.[10-12]

Psoralen derivatives

The 0·3% 8MOP ointment and lotion were purchased from Taisho Pharmaceutical Co., Tokyo, Japan. The 2% 3CPs ointment and 0·01 M MPP ointment were kindly provided by Dr L. Dubertret. TMA was a gift from Dr F. Dall'Acqua, and was prepared as a 0·1% lotion.

Treatment

Topical PUVA therapy was performed according to our standard protocol.[13,14] In brief, 2 h after the topical application of one of the psoralen preparations, the patients were irradiated with UVA. The initial UVA dose

Correspondence: Dr Akira Takashima, Department of Dermatology, The University of Texas Southwestern Medical Center at Dallas, 5323 Harry Hines Blvd, Dallas, TX 75235-9069, U.S.A.

for 8MOP was one-third of the minimal erythema dose (MED), and the dose was subsequently increased to one-half MED, 1 MED, 2 MED and 3 MED. In topical PUVA using 3CPs, MPP or TMA, the UVA irradiation was started at 1–5 J/cm^2 and increased to 10 J/cm^2.[10–12] Treatment was given from one to three times a week. None of the patients received any other treatment, except for etretinate, antihistamines and the topical application of white petrolatum.

Measurement of minimal erythema dose (MED)

Two hours after topical application of a psoralen preparation to uninvolved skin on the back, UVA irradiation was given at doses ranging from 0·03 to 15 J/cm^2. The MED was assessed 2 days after irradiation of the skin.

Patch testing, photopatch testing and photopatch test mapping

Psoralen derivatives were diluted in acetone or white petrolatum and applied to the uninvolved skin on the back using Finn chambers. The patch tests were removed after 48 h and the reaction evaluated at both 48 h and 72 h. For the photopatch tests, the patches were removed after 24 h and the test sites immediately irradiated with UVA (0·3 J/cm^2) using window glass as a filter. Compounds serially diluted were applied and graduated doses of UVA given in photopatch test mapping, so as to differentiate photoallergic from phototoxic reactions. The skin reactions were evaluated at 24 and 48 h following UVA exposure and were scored according to the ICDRG standards.

Light sources

The light sources used for PUVA treatment and phototesting were UVA fluorescent lamps, FL20S.BL and FL32S.BL (Toshiba, Tokyo). The irradiance at the skin surface was 5–10 mW/cm^2 as measured with a UVA radiometer (UVR 305/365.D, Eisai-Toshiba, Tokyo). This has been described elsewhere.[12,14]

Immunohistological studies

Cryostat sections of biopsies were processed for routine immunoenzymatic staining. The antibodies and reagents used were OKT3, OKT4, OKT8 and B2 (Ortho Diagnostics, Raritan, NJ, U.S.A.) together with the avidin/biotin/peroxidase staining kit (Lipshaw Corporation, Detroit, MI, U.S.A.).

Results

Results of phototesting and diagnosis

Case 1. A 44-year-old female patient with generalized pustular psoriasis had PUVA treatment with oral 8MOP for 5 years and subsequently received topical PUVA treatment. After 99 treatments with topical PUVA, the patient developed a vesicular itchy dermatitis in the treated areas. This occurred 24 h after PUVA and cleared rapidly with the application of a topical corticosteroid.

In this patient the patch tests were negative for all of the psoralens and on photopatch testing there was a strong reaction to 8MOP and positive reactions were also observed to 3CPs, that had never been applied to this patient (Table 1). Patch testing with 8MOP after pre-

Table 1. Results of patch and photopatch testing of psoralen derivatives*

Psoralen	Concentration (%)	Case 1		Case 2		Case 3		Case 4	
		PT†	PPT‡	PT	PPT	PT	PPT	PT	PPT
8MOP	10^{-1}	—	++	+	+	—	—(++)§	—	—
3CPs	10^{-1}	—	+	—	—	—	++	—	—
MPP	10^{-1}	—	—	—	—			—	—
TMA	10^{-1}			—	—			—	++
TMP¶	10^{-1}	—	—	+	+	—	±	—	±

*Data shown are the results at 48 h.

†Patch test.

‡Photopatch test. A dose of 0·3 J/cm^2 of UVA was given through a window-glass filter. Data shown are the skin reactions scored according to the ICDRG standard.

§This is the result after sensitization to 8MOP

¶Trimethylpsoralen.

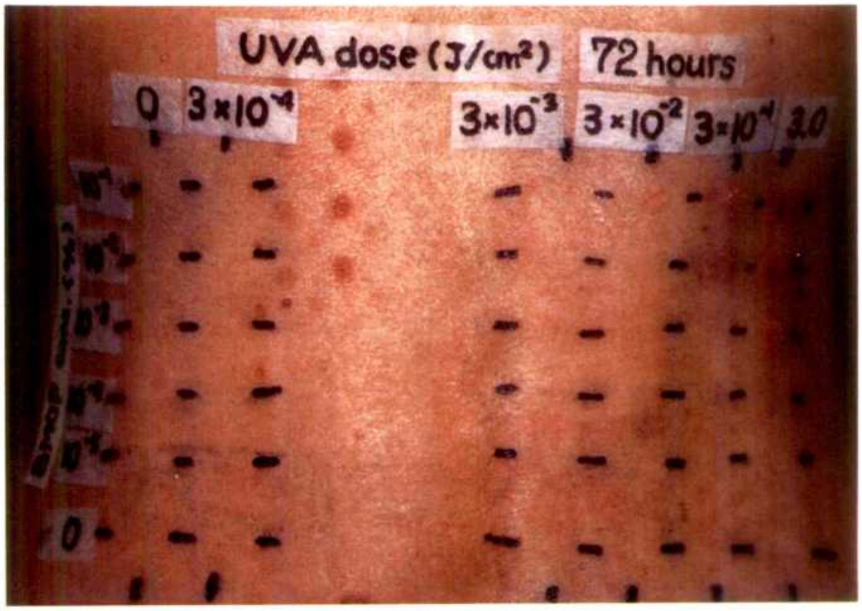

Figure 1. Photopatch test mapping for 8MOP 48 h after UVA exposure on the back.

irradiation with UVA failed to show a positive reaction. To differentiate between photoallergic and phototoxic reactions to 8MOP, photopatch test mapping was performed. This patient demonstrated positive reactions with 8MOP concentrations down to 10^{-3}% and UVA doses down to 3×10^{-2} J/cm^2 (Fig. 1). This patient was more than 10 times more sensitive to 8MOP than controls. The diagnosis of allergic photocontact dermatitis due to 8MOP was made with a cross-reaction to 3CPs.

Case 2. This 63-year-old male had been on treatment with topical 8MOP for 8 months. After a total of 33 treatments he developed an acute itchy dermatitis in the PUVA-treated areas. This occurred within 24 h after therapy.

Patch tests performed on this patient were positive to 8MOP and to 4,5′,8-trimethylpsoralen (TMP) that had never been applied to this patient (Table 1). Photopatch test mapping was carried out to determine whether or not there was a coexisting photoallergy (Table 2). UVA irradiation with doses lower than 3×10^{-1} J/cm^2 failed to augment the contact allergic reaction, indicating that photoallergy was not involved. A diagnosis of allergic contact dermatitis due to 8MOP with a cross-reaction to TMP was thought to be most likely in this case.

Case 3. A 65-year-old female patient with psoriasis developed acute dermatitis in the treated area after seven treatments with topical 3CPs PUVA. On discontinuing the treatment the dermatitis cleared. Two years later,

Table 2. Results of photopatch test mapping for 8MOP in Case 2*

UVA dose (J/cm^2)	8MOP concentration (%)				
	10^{-5}	10^{-4}	10^{-3}	10^{-2}	10^{-1}
3	—	—	+	+ +	+ +
3×10^{-1}	—	—	—	+	+
3×10^{-2}	—	—	—	+	+
3×10^{-3}	—	—	—	+	+
0	—	—	—	+	+

*Data shown are the results at 48 h.

†The broken line indicates the range of concentrations in which positive reactions up to (+) were seen in two out of 10 healthy volunteers.

Table 3. Results of photopatch test mapping for 3CPs in Case 3*

UVA dose (J/cm^2)	3CPs concentration (%)			
	2×10^{-3}	2×10^{-2}	2×10^{-1}	2
5	—	+	+ +	+ +
1	—	+	+ +†	+ +
0·5	—	+	+ +	+ +
0·1	—	—	+	+ +
0	—	—	—	—

*Data shown are the results on day 3.

†None of the five healthy volunteers examined showed a positive reaction to the combination of 2×10^{-1}% 3CPs and 1 J/cm^2 of UVA.

Table 4. Comparison of reactions in photopatch test mapping for 8MOP before and after sensitization in Case 3[*]

UVA dose (J/cm^2)	8MOP concentration (%)			
	3×10^{-4}	1×10^{-3}	1×10^{-2}	3×10^{-1}
2·4	—[†](—)[‡]	++(—)	++(+)	++(++)
1·2	— (—)	+ (—)	++(—)	++(—)
0·6	— (—)	++(—)	++(—)	++(—)
0·3	— (—)	++(—)	++(—)	++(—)
0 15	— (—)	++(—)	++(—)	++(—)
0	— (—)	— (—)	— (—)	— (—)

[*] Data shown are the results at 48 h.

[†] Results after sensitization to 8MOP.

[‡] Results in parentheses show the reactions prior to topical PUVA treatment.

topical PUVA with 8MOP was given to this patient and she developed a similar dermatitis after only nine treatments.

This patient gave a positive reaction to 3CPs in the photopatch test but not in the normal patch test (Table 1). Photopatch test mapping for 3CPs showed that she was highly reactive and had the typical pattern of a photocontact allergy (Table 3). At that time there was no cross-reaction for 8MOP, but subsequently when the patient was examined after the development of hypersensitivity to 8MOP 2 years later, photocontact allergy to 8MOP was found (Tables 1 and 4). The MED for 8MOP had decreased from 1·1 to 0·06 J/cm^2 UVA after she had become sensitized to this compound.

Case 4. This was a 33-year-old female patient with psoriasis who developed itchy oedematous erythema in the treated area following 10 treatments with topical TMA and after a total UVA dose of 81 J/cm^2. This reaction developed 12 h after her last PUVA treatment.

Patch tests carried out with psoralen derivatives were all negative but photopatch testing gave a positive reaction to TMA (Table 1). However, patch testing with TMA after pre-irradiation with UVA failed to give a positive reaction. No cross-reaction was observed to any of the other psoralen derivatives tested. Photopatch test mapping for TMA in this patient demonstrated hypersensitivity to the compound with a photoallergic pattern as shown in Table 5. In addition, the MED for TMA was over 15 J/cm^2 before starting TMA-PUVA and decreased to 0·3 J/cm^2 after sensitization. In this patient a diagnosis of allergic photocontact dermatitis was made.

Table 5. Results of photopatch test mapping for TMA in Case 4[*]

UVA dose (J/cm^2)	TMA concentration (%)				
	10^{-5}	10^{-4}	10^{-3}	10^{-2}	10^{-1}
3	—	+	+	++	++
3×10^{-1}	—	—	+	++	++[†]
3×10^{-2}	—	—	—	+	+
3×10^{-3}	—	—	—	—	—
0	—	—	—	—	—

[*] Data shown are the results at 48 h.

[†] None of the four healthy volunteers examined showed a positive reaction to the combination of 10^{-1}% TMA and 0·3 J/cm^2 of UVA.

Histological findings

Biopsies of the skin were taken from cases 1, 3 and 4 of the patients with photoallergic contact dermatitis. The histology showed spongiosis with a lymphocytic infiltrate in the upper dermis in addition to the psoriatic changes. A biopsy was also taken from an experimentally induced photoallergic dermatitis after photopatch testing with TMA in a patient who was sensitive to this psoralen. There was a dense lymphocytic infiltrate around blood vessels in the upper dermis and spongiosis of vesicles in the epidermis (Fig. 2a). Immunohistological studies revealed that the majority of the infiltrating cells were T lymphocytes and CD4 positive cells were more common than CD8 positive cells (Figs 2b and c).

Incidence of delayed hypersensitivity to psoralens in topical PUVA therapy

Of 371 patients with psoriasis treated with topical 8MOP during PUVA treatment in our clinic, we have observed contact allergy to 8MOP in only one patient (Case 2) and photocontact allergy in two of the patients (Cases 1 and 3). These patients developed a delayed hypersensitivity after a relatively long period, ranging 8–36 months with a mean of 23 months (Table 6). Compared with 8MOP, both 3CPs and TMA (monofunctional psoralens) have a much higher antigenic capacity. Ten patients with psoriasis were treated with PUVA with topical 3CPs and four patients developed an allergic contact and/or photocontact dermatitis.[10] TMA-induced allergic photocontact dermatitis occurred in six of the 17 patients treated with this compound. Another monofunctional psoralen, MPP, caused no allergic reactions in the seven patients who were treated with this compound.[12]

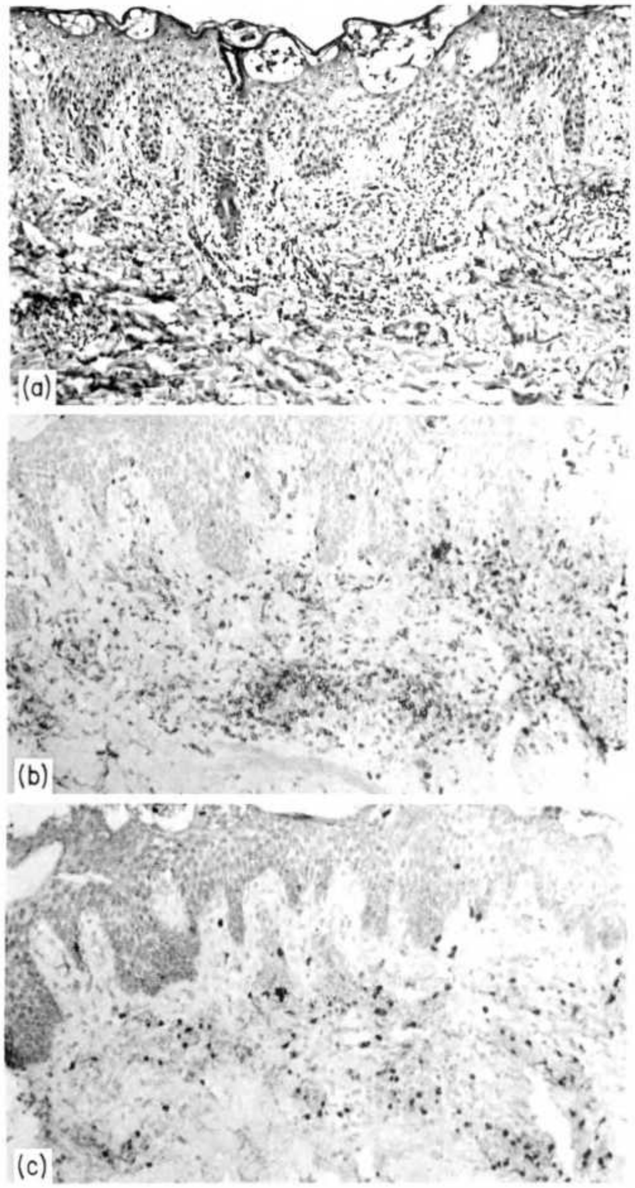

Figure 2. Histology of a positive photopatch test to 0·1% TMA 48 h after UVA exposure. Section stained with (a) haematoxylin and eosin; (b) OKT4 and (c) OKT8 (× 100).

Discussion

It is generally considered to be difficult to distinguish between allergic and toxic photoreactions to phototoxic compounds such as psoralen. However, we were able to make a definite diagnosis of photocontact allergy to psoralen in our patients. They suddenly developed an acute dermatitis after being given PUVA and had positive photopatch tests. Cross-reactions to other psoralens derivatives were seen in some of the patients. Also, the histological features seen in our cases were almost identical to those seen in allergic contact dermatitis. These findings appear to fulfill the criteria for photoallergy.[15,16]

Several authors have reported photoallergy to psoralens. Sidi and Bourgeois-Gavardin first reported five cases of allergic photocontact dermatitis to 8MOP used for the treatment of vitiligo, but the results of the phototesting were not given in detail.[2] Photoallergy to 8MOP was induced in three volunteers under experimental conditions, with positive photopatch test reactions using 0·1% 8MOP.[3] A case of photoallergic dermatitis from orally administered 8MOP in a patient on PUVA for psoriasis was reported and in this case relatively small doses of UVA were sufficient to produce photoallergic reactions with topical and oral 8MOP.[4] A case was reported of allergic photocontact dermatitis due to 8MOP, 5-methoxypsoralen and imperatorin from plant sources and a concentration as low as 10^{-4}% for 8MOP induced allergic reaction on photopatch testing.[5] In our studies we found that photopatch test mapping clearly demarcated the limits of phototoxic reactions to psoralens by applying serially diluted samples and giving graduated doses of UVA. Reactions occurring beyond these limits were considered to be of an allergic nature. These photopatch test mapping studies were of considerable value in distinguishing photoallergic from phototoxic reactions.

The histopathological findings in our results also

Table 6. Comparison of antigenic potencies of four psoralen derivatives

	8MOP	3CPs	TMA	MPP
Number of patients treated	371	10	17	7
Allergic reaction				
Contact	1	—	—	—
Photocontact	2	2	6	
Contact and photocontact	—	1	—	—
Undetermined	—	1	—	—
Total (%)	3 (0·8)	4 (40)	6 (35·3)	0
Time until onset (mean)	8–36 months (23 months)	10–50 days (24 days)	14–98 days (34 days)	

favoured the diagnosis of allergic photocontact dermatitis rather than phototoxicity.[15] The spongiosis of the epidermis with a perivascular lymphocytic infiltrate is similar to that previously reported in cases in which there has been photoallergy to psoralens.[3–5] Our immunohistological studies demonstrated that CD4 positive (helper/inducer lymphocytes) were predominant in this reaction and this is in agreement with previous studies on allergic contact dermatitis.[17,18]

One of our patients (Case 2) had a positive reaction to 8MOP on patch testing without photoaugmentation (Tables 1 and 2). The incidence of contact allergy to 8MOP appears to be low because only one of the 371 patients developed such a reaction, and there are only a few reports in the literature.[19,20] The antigenic potency of 8MOP is low as evidenced using a human assay in the maximization test.[21]

The advantage of using monofunctional psoralens in PUVA therapy is the lower risk of skin cancers due to their limited carcinogenic and/or mutagenic potency.[7,8] We previously reported that TMA, a monofunctional compound, possesses far less phototoxicity and a higher therapeutic efficacy for psoriasis as compared with 8MOP.[11] However, this study has shown that both 3CPs and TMA have a significantly higher antigenic potency than 8MOP. New monofunctional derivatives are necessary that are less antigenic and more effective than 8MOP.

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Cyclosporin A in atopic dermatitis: therapeutic response is dissociated from effects on allergic reactions

C.S.MUNRO, ELIZABETH M.HIGGINS, JANET M.MARKS, B.MARTINA DALY,
P.S.FRIEDMANN AND S.SHUSTER

Department of Dermatology, Royal Victoria Infirmary, Newcastle upon Tyne NE1 4LP, U.K

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Summary Fourteen patients with severe chronic atopic dermatitis were treated with cyclosporin A (CyA, Sandimmun®; 5 mg/kg/day) for 7–16 weeks. All showed a marked clinical improvement and half could omit topical corticosteroid treatment during therapy. Adverse effects were minor, but two patients relapsed despite continued treatment. In the others, the disease recurred soon after stopping CyA. Serum IgE levels and prick-test responses were unchanged by CyA. Immediate and late-phase cutaneous responses to intradermal house dust mite antigen (HDM) were significantly increased during treatment; but a delayed response, present at 24 and 48 h, was unaffected. Four of six patients challenged with HDM patch tests to tape-stripped skin during treatment showed eczematous reactions at 48 h. Thus, cyclosporin A has a powerful therapeutic effect in atopic dermatitis but does not reduce allergic responses to inhalant antigens.

Cyclosporin A (CyA), an immunosuppressive agent, has been found to be effective in various dermatoses.[1–6] Recent reports of a few patients indicate that atopic dermatitis also responds to this drug.[7–9] We have treated a larger group of patients with severe chronic atopic dermatitis with CyA, in order to explore its efficacy further. We have also explored its possible mechanism of action in the disease by examining the effect of CyA on the abnormal allergic responses to environmental antigens, such as house-dust-mite antigen (HDM), characteristic of atopic individuals. A causal link between such hypersensitivity reactions and atopic dermatitis has long been suspected but is not proven.[10,11] If they are indeed relevant to dermatitis, drugs which improve the disease might be expected also to reduce hypersensitivity reactions. We therefore measured the effect of CyA treatment on the response to HDM, using both intradermal tests to follow immediate, late-phase (6 h) and delayed (48 h) reactions, and epicutaneous patch tests on tape-stripped skin, which were read at 48 h. While CyA greatly improved atopic dermatitis, we found no reduction in any component of the response to HDM, and immediate and late-phase responses were paradoxically increased.

Methods

Fourteen patients with chronic atopic dermatitis, severe enough to require continuous use of potent topical corticosteroids, were recruited in an open therapeutic trial of cyclosporin A (Sandiman, Sandoz). All patients gave informed consent. Patients with abnormal renal or hepatic function were excluded. There were eight male and six female patients, and the mean age was 28·6 (range 17–56) years. Each patient was treated with CyA at a dose of 5 mg/kg/day, given as a single evening dose, for periods of up to 16 weeks, at the end of which time the drug was either discontinued abruptly (in nine patients) or reduced gradually (in five). Patients were allowed to continue using topical steroid treatment as necessary, and no other changes were made. Two additional atopic patients (one male, one female, ages 18 and 25) were studied from a group being treated with CyA (5 mg/kg/day) for alopecia areata; they had no history of dermatitis but were sensitive to HDM in skin prick tests. Blood pressure, and standard biochemical indicators of renal and hepatic function were monitored throughout. Whole blood cyclosporin levels were measured 12–14 h after the oral dose, using the specific monoclonal antibody RIA kit (Sandoz).

The response to treatment was evaluated by clinical assessment of erythema, weeping, scale, excoriation and

Correspondence: Dr C.S.Munro.

lichenification. Severity of itching and requirement for topical steroids were reported by the patients. In 11 of the 14 patients, the area involved by active dermatitis was assessed at each clinic visit using the rule of nines. In eight patients, severity of lesional erythema, scale, and excoriation was scored on a 0–3 scale in each of four areas (head and neck, trunk, arms, and legs), giving a maximum score of 12 for each parameter.

Components of the atopic allergic response were measured before treatment, and again after 4–6 weeks of treatment. In 10 patients, total serum IgE levels were measured by routine laboratory ELISA before and after 6 weeks of treatment with CyA. Skin testing of patients was approved by the hospital ethical committee. Immediate hypersensitivity responses were elicited with a range of common antigens (Bencard) as prick tests. Responses were considered positive if the weal diameter was more than 3 mm greater than the control at 15 min. The full time course of the response to intradermal injection of HDM was followed in six patients with dermatitis and the two with alopecia areata, all of whom displayed immediate hypersensitivity to the antigen. HDM antigen (Pharmacia) was injected at doses of 1, 5, and 25 units in 0·05 ml saline and the response was measured as the change in skinfold thickness with Harpenden callipers[12,13] at intervals of 15 min for an hour, then at 6, 24 and 48 h. In six patients, these measurements were repeated on a third occasion, 6 weeks after treatment had been discontinued. Epicutaneous challenges with HDM ($1{\cdot}25 \times 10^4$ units in 25 μl) and diluent control were made in six patients during CyA treatment. Solutions were applied, on patch test felts (Al-test), to clinically normal back skin which had been stripped with cellophane tape until the first glistening points appeared. After removal of patch tests at 48 h, the responses were assessed according to conventional patch-testing criteria.[14]

Statistics

The difference between individual measurements in paired data was compared using *t*-tests. Two-way within-patient analysis of variance was used to compare responses to intradermal HDM. In order to avoid multiple comparisons of related data, the immediate response was characterized by deriving a single statistic, the area under the curve, from measurements between 15 and 60 min. The measurements at 6, 24 and 48 h, which may represent distinct responses, were analysed individually.

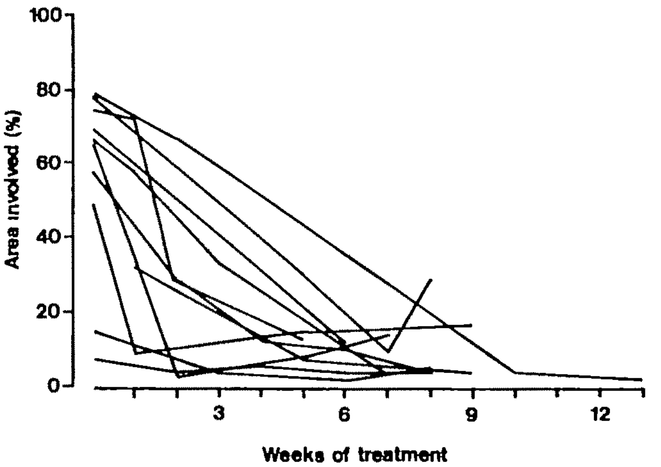

Figure 1. The effect of cyclosporin A treatment on the extent of active eczema, as a percentage of body surface area, in 11 patients.

Results

Clinical effects

All patients showed clinical benefit within 3 weeks. The earliest changes, often apparent within the first week, were lessening of itching and erythema of the lesions. Improvement in weeping, excoriation and scaling followed. Lichenification, when present, showed a more gradual response over the period of the study (up to 16 weeks). All patients reported a marked reduction in their requirement for topical steroid application during CyA treatment. Seven patients required emollients only after remission was induced, and the other patients reported that they could use less potent steroids, or apply them less frequently or to smaller areas, than before treatment. The effect of CyA treatment on the area involved in 11 individual patients, assessed by the rule of nines, is shown in Figure 1. The mean body area involved at start of treatment was 50·3% (range 6–81%); at 6 weeks the mean area was 9·9% (range 2–35%), a significant reduction ($P<0{\cdot}01$). Scores for erythema, scale and excoriation in eight patients, who included two undergoing relapse at 6 weeks, are shown in Table 1.

Mild fluctuations in the activity of the dermatitis occurred during CyA treatment in several patients, but in two patients the rash worsened and, because of the possibility that CyA was aggravating the disease, treatment was stopped after 7 and 8 weeks. In one of these patients the relapse was on the face and upper chest and resembled seborrhoeic dermatitis. It resolved on stopping CyA treatment (see below), but the atopic dermatitis worsened, as it did also on stopping CyA in the second patient. In five of the patients who responded well to the

Table 1. Clinical effects of CyA treatment in eight patients

Weeks	Erythema	Scale	Excoriation
Pre-CyA	6·4±0·8	3·3±1·9	5·5±2·9
2	4·3±1·4***	1·5±1·5*	2 1±2·1**
6	4·0±3·0*	2·2±2·0	2·1±2 3**

Values given as mean and standard deviation.
*$P<0{\cdot}05$; **$P<0{\cdot}01$; ***$P<0{\cdot}001$ compared with pre-CyA values by paired *t*-test.

drug, the dose of CyA was gradually reduced after 5–14 weeks, and then stopped. Dermatitis reappeared in all these patients as the dose was reduced, but even with 1–2 mg/kg/day CyA the rash was better controlled than before treatment. Of the two patients with alopecia areata, one showed complete regrowth, which persisted after treatment was stopped, but in the other the drug had no effect.

There were no major adverse effects. Paraesthesiae, initial nausea and diarrhoea, and a septic folliculitis were noted, each in one patient; several developed mild hypertrichosis. One woman said her co-ordination was subtly impaired, with a deterioration in her golf game and difficulty with typing. One female patient developed a recurrence of perineal warts, and in another they occurred for the first time during CyA treatment. One patient developed seborrhoeic dermatitis which responded to topical ketoconazole; when it recurred, CyA was stopped to see whether it was related to treatment: the seborrhoeic dermatitis improved, but the atopic dermatitis relapsed.

The mean whole blood CyA concentration during treatment was 224 (±SD 97) ng/ml. There was no evidence of disturbed hepatic or haematological function. There was no change in mean blood pressure or mean serum creatinine, but the mean serum urate rose by 0·03±0·04 mmol/l, which was significant ($P<0{\cdot}05$). In three men serum urate was above the normal range (0·15–0·42 mmol/l) at the end of full dose treatment. In two of these, serum creatinine had also risen, to 109 and 110 μmol/l (laboratory normal range 62–105 μmol/l); blood pressure had risen from 130/80 to 135/95 and from 135/85 to 145/95, respectively. In all cases the abnormal findings returned to baseline values following cessation of CyA treatment.

Effect of atopic responses

Serum IgE levels were unchanged by treatment. The geometric mean total serum IgE in 10 patients with atopic dermatitis was 1295 units (range 69–71,000) before treatment and 1292 units (range 91–8000) after 6 weeks' treatment with CyA. When immediate hypersensitivity was examined by standard skin prick tests, all control positive responses remained positive during treatment.

The responses to intradermal HDM in untreated patients were an immediate weal and flare reaction, followed by persistent erythema and increased skinfold thickness. The latter was dose-dependent in the first hour and at 6, 24 and 48 h (Fig. 2a). During CyA treatment (Fig. 2b), despite the good clinical response of atopic dermatitis, immediate weal formation was increased. This treatment effect was significant ($P<0{\cdot}05$) when the summarized responses over the first

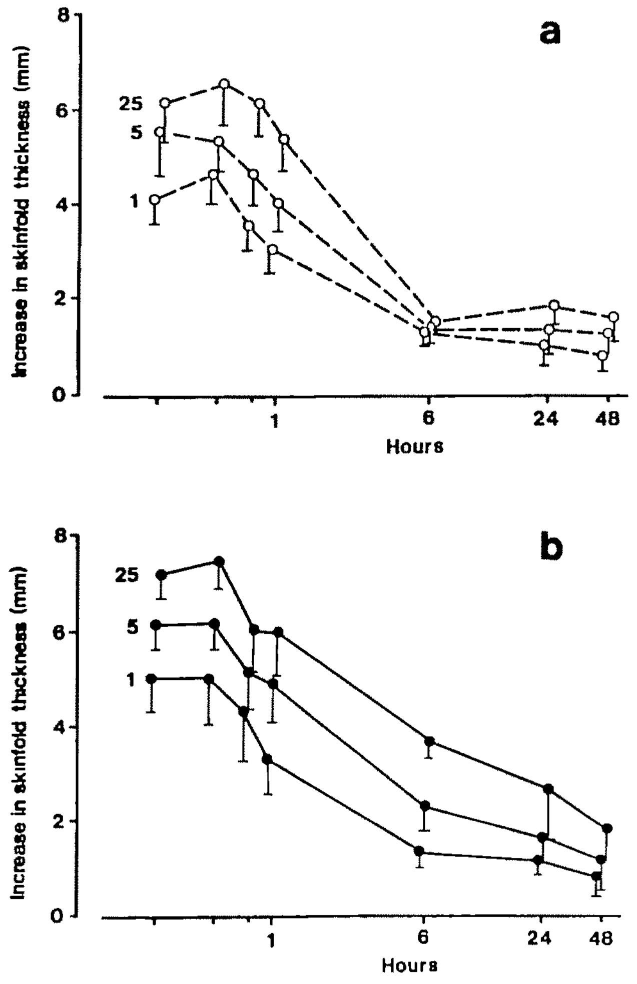

Figure 2. The oedema response to intradermal challenge with house-dust-mite antigen in eight patients (a) before and (b) during treatment with CyA. Units of antigen injected are shown on the left, and the measured increase in skinfold thickness over baseline is shown as mean and standard error at each time point.

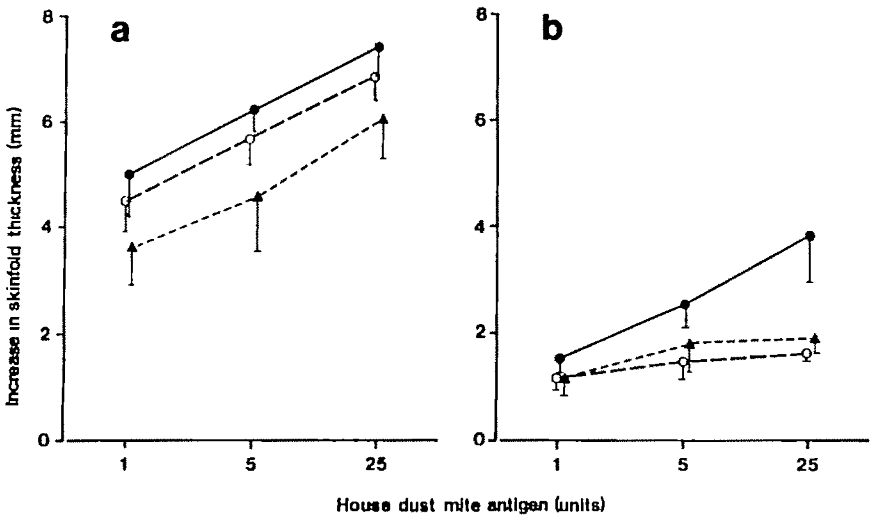

Figure 3. The response to intradermal house dust mite antigen in six patients before (—○—), during (—●—), and after (--▲--) CyA treatment. Dose response curves at (a) 30 min and (b) 6 h are shown. Values are mean and standard error.

hour were compared. The late phase (6 h) response was also greater on CyA ($P<0{\cdot}02$). The effect at 6 h was more marked at higher antigen doses, and the altered slope of the dose response curve was significant ($P<0{\cdot}05$). However, the responses at 24 and 48 h were not significantly affected. The increases in immediate and late-phase responses were not due to repeated testing, since in six patients tested for a third time after treatment, pre- and post-treatment responses were comparable (Fig. 3).

In the six patients with atopic dermatitis to whom HDM was applied as an epicutaneous patch test on tape-stripped skin during CyA treatment, eczematous responses, with erythema and palpable induration, developed in four and were equivocal in the other two. Reactions to diluent control were all negative.

Discussion

We have provided further evidence that CyA, at a dose of 5 mg/kg/day, is an effective oral agent for controlling severe atopic dermatitis. It rapidly improves symptoms, notably itch, and in half the patients topical steroids could be stopped altogether during CyA treatment. Paraesthesia, gastro-intestinal upset and hypertrichosis were encountered but did not necessitate cessation of therapy, and changes in renal function which occurred in three patients resolved on stopping the drug. The appearance of genital warts may reflect the immunosuppressive effect of the drug, as may seborrhoeic dermatitis, since the latter is a common complication of the acquired immunodeficiency syndrome, in which $CD4^+$ T-cell function is impaired.[15] In most patients the dermatitis relapsed within a few days after treatment was stopped but in five patients in whom the dose was reduced in stages, 1–2 mg/kg/day maintained better control of the dermatitis than topical corticosteroids had done before CyA treatment. Four of nine patients in whom CyA was stopped abruptly developed widespread dermatitis after treatment was discontinued. These included the patient who developed seborrhoeic dermatitis and another whose atopic dermatitis relapsed during treatment, but all four had had extensive disease (area affected 53–87%) before treatment. Further study is required to establish dosage regimes including optimal dosage for control, maintenance, and withdrawal of treatment.

In examining possible mechanisms by which CyA may control dermatitis, we found that serum IgE, and IgE-mediated immediate hypersensitivity responses in skin-prick tests were not suppressed; indeed the immediate response to intradermal HDM was increased. We have previously shown in these and other patients being treated with CyA[16] that treatment does not augment the response to histamine, nor histamine release from mast cells as measured by the response to compound 48/80. We can also exclude the possibility that the increase in wealing responses in atopic dermatitis is due to loss of tachyphylaxis (histamine release being reduced when the dermatitis is controlled), since the findings were identical in the two atopic patients with alopecia areata but not dermatitis. Thus, the augmentation by CyA of immediate reactions to antigen in atopics suggests either increased release, or an increased response to the non-histamine component of the reaction.

The late-phase cutaneous response to intradermal antigen challenge[17] was also increased by CyA, and reverted to pretreatment levels when treatment was stopped. Although late phase (6 h) responses to house dust mite occur in association with large immediate reactions,[18,19] they are not dependent on the effects of histamine.[20] Unlike the immediate reaction, the augmentation of the late-phase response by CyA was greatest at the highest antigen dose. This alteration in the slope of the dose–response curve suggests that the effect of CyA on the late-phase response involves a qualitative change, rather than a simple increase in mediator release. Thus CyA may modify the effects of non-histaminic mediators released in response to antigen challenge.

The cutaneous response to intradermal HDM was still evident at up to 48 h, which is consistent with the hypothesis that in atopy, cell-mediated immunity to inhalant antigens may coexist with immediate sensitivity.[11,20–23] It has been suggested that such delayed responses to house dust mite may contribute to the pathogenesis of atopic dermatitis.[22,23] Delayed reactions can also be induced by house dust mite applied in epicutaneous tests,[22,23] and such responses were induced in four out of six of our CyA treated patients, two more giving equivocal responses. We have previously obtained similar responses in untreated patients with atopic dermatitis (unpublished data). Although the immunosuppressive effects of CyA appear to be principally on the induction of cell-mediated immunity,[24] delayed hypersensitivity to common contact sensitizers elicited by patch testing in patients with contact dermatitis is suppressed by oral CyA given at 5 mg/kg (J.McLelland, P.S.Friedmann, data submitted for publication). We cannot in these few patients exclude a partial or dose-related effect of CyA on the response to house dust mite. Nevertheless, contact hypersensitivity reactions differ histologically from delayed reactions to house dust mite,[22] and it is possible that the recognized effects of CyA on immune system[24] are not relevant to the latter. The fact that CyA produces a striking improvement in the dermatitis without significantly reducing the delayed responses to intradermal or epicutaneous testing must therefore open to question the relationship of these reactions to atopic dermatitis.

We have shown that CyA has a powerful therapeutic effect in atopic dermatitis, yet paradoxically it increases immediate and late phase hypersensitivity and leaves delayed responses apparently unchanged. Unless the action of CyA is upon secondary mechanisms triggered by these responses, it follows that none of them are directly relevant to the pathogenesis of the disease. This surprising conclusion requires confirmation by other means. Regardless of its mechanism of action, however, the considerable improvement CyA produces in atopic dermatitis should encourage further investigation of its potential in the management of the disease.

Acknowledgments

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'High single-dose' European PUVA regimen also causes an excess of non-melanoma skin cancer

I.BRUYNZEEL, W.BERGMAN, H.M.HARTEVELT, C.C.A.KENTER, E.A.VAN DE VELDE,* A.A.SCHOTHORST AND D.SUURMOND

*Departments of Dermatology and *Medical Statistics*
University Medical Center, Leiden, The Netherlands

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Summary We report the results of a long-term (12·8 years) follow-up study of the detection of malignant and benign skin tumours in patients with psoriasis, who were treated with PUVA according to the European, 'high single-dose' regimen. A total of 13 squamous cell carcinomas (SCC) and 24 basal cell carcinomas (BCC) were diagnosed in 11 of 260 patients. The incidence of both SCC and BCC was increased in comparison with the general Dutch population. The ratio of SCC to BCC in the general population was 1:8 but was 1:2·5 in our study group. A positive correlation was observed between the development of SCC and the total UVA dosage, the age of the patient at the start of the PUVA treatment and a history of arsenic use. This dose-related increase in the incidence of SCC, reported in studies from the U.S.A., has not been found in earlier European studies. The average time period between the start of PUVA therapy and the diagnosis of the first malignant skin tumour was 6·0 years for SCC and 4·7 years for BCC. Among the 49 benign skin tumours were actinic keratoses, a kerato-acanthoma and 'PUVA keratoses', a newly described hyperkeratotic lesion, especially found in PUVA-treated patients.

Since the introduction of PUVA treatment for patients with psoriasis, four large co-operative groups have undertaken long-term follow-up studies for the detection of non-melanoma skin cancer and have published their early findings[1–4] and recent updates.[5–9] Seven other centres have also reported their experience.[10–15] The reports from the U.S.A. all described an increased incidence of squamous cell carcinoma (SCC).[5,6,9] One of the American groups showed a substantial dose-related increase, suggesting that PUVA is a primary carcinogen.[5,6] This is in contrast with most of the European studies, which only reported an increased incidence of SCC related to previous exposure to other carcinogens[7,8,11] or no increased incidence at all.[10,12–15] In Europe, a PUVA schedule is used which provides for fewer, but higher, single-dose treatments as compared to the schedule used in the U.S.A. This might explain the different skin cancer risks found in the U.S.A. and in Europe.

We present our one-centre retrospective study of 260 patients with psoriasis treated with the 'high single-dose' European schedule of PUVA therapy after an average follow-up period of 104 months.

Methods

In the period between 1975 and 1988, 334 patients with psoriasis were treated with oral photochemotherapy (PUVA) in our clinic. Preliminary results of this group of patients have been reported earlier.[16] Excluded from the present study were 14 patients who received less than 20 J/cm^2 UVA, six patients who were treated locally, mainly on their hands and feet, and 21 patients who died before the first follow-up screening. To our knowledge, all 21 patients died from causes unrelated to PUVA treatment or psoriasis. Thirty-three patients (10%) were lost from follow-up or declined examination.

Patient data recorded included sex, age, skin type, previous use of arsenicals, methotrexate (MTX), etretinate, a history of skin cancer, exposure to X-rays or UVB and details of PUVA therapy. Further treatment with UVB during the follow-up period was also recorded. A total of 260 patients were examined, 165 males and 95 females. Their mean age at the start of PUVA treatment was 43·4 years, range 20–86 years. No patients with

Correspondence: Dr W.Bergman, Department of Dermatology, University Hospital, P.O. Box 9600, 2300 RC Leiden, The Netherlands.

skin type I were treated, 17 had skin type II, 213 skin type III, 21 skin type IV and nine skin type V. Skin types were defined according to Johnson and Lookingbill.[17] Two patients had a previous history of basal cell carcinoma (BCC), 24 were treated earlier with arsenicals, 33 with MTX and two patients had a history of X-ray therapy. Etretinate was used by 56 patients and 108 patients were treated subsequently with UVB. Since 1984 UVB has been the treatment of first choice in our clinic. With regard to the short period of follow-up of UVB treatment, we did not study the skin cancer risk of UVB and PUVA separately.

All patients were treated with a standard PUVA regimen. 8-Methoxypsoralen (0·5–0·6 mg/kg body weight) was taken orally 2 h before treatment. The initial UVA dose of each patient depended on the results of the determination of the skin sensitivity to UVA,[18] and ranged from 0·3 to 1·0 J/cm^2. The clearing treatment consisted of a three times a week schedule, in which the dose was elevated by 10, 20 or 30% every other session. Immediately after clearing, a maintenance regimen with longer treatment intervals followed, during which period the frequency was gradually lowered to one treatment every 2 or 3 weeks. The last administered UVA dose was kept unaltered, ranging from 2 to 15 J/cm^2. If a patient relapsed, the treatment frequency was temporarily increased. The individual cumulative UVA dose varied from 22 to 4331 J/cm^2 and the average cumulative UVA dose was 824 J/cm^2. Details of PUVA treatment are listed in Table 1.

Patients were examined for the presence of malignant and benign skin tumours, and PUVA-induced freckles. The diagnosis of SCC, BCC and kerato-acanthoma was confirmed histologically in all cases. The diagnosis of 'PUVA keratosis', a hyperkeratotic papule with a warty aspect, is based on a distinct histological picture (Prof. E. Scheffer, Department of Pathology). There is a broad papillomatous epidermis showing acanthosis, hyper- and parakeratosis and some apoptotic cells and in the papillary dermis a mild perivascular inflammatory infiltrate, slightly invading the epidermis (Figs. 1 and 2).

Table 1. Data on PUVA therapy: total number of treatments, total UVA dosage and the corresponding numbers of patients ($n = 260$)

Total number of treatments	Number of patients	Total UVA dosage (J/cm^2)	Number of patients
<50	55	<200	56
50–99	86	200– 499	61
100–149	68	500– 999	58
150–199	33	1000–1499	41
>200	18	1500–1999	22
		>2000	22

Actinic keratosis was usually diagnosed on its clinical appearance and histopathological confirmation was available in 20% of the lesions.

Statistical methods

For statistical analysis the number of person years at risk was computed.[19] The number of person years was calculated between arbitrarily chosen opening and closing dates. The date of the first PUVA treatment was used as the opening date, and for the closing date we used the date of diagnosis of the first skin malignancy or the end of the study, namely 31 December 1987. The incidence per 100,000 person years of the risk of developing a SCC or a BCC was computed for the overall follow-up time and for the periods of 0–4, 5–9 and 10–14 years after the start of PUVA therapy. The incidence

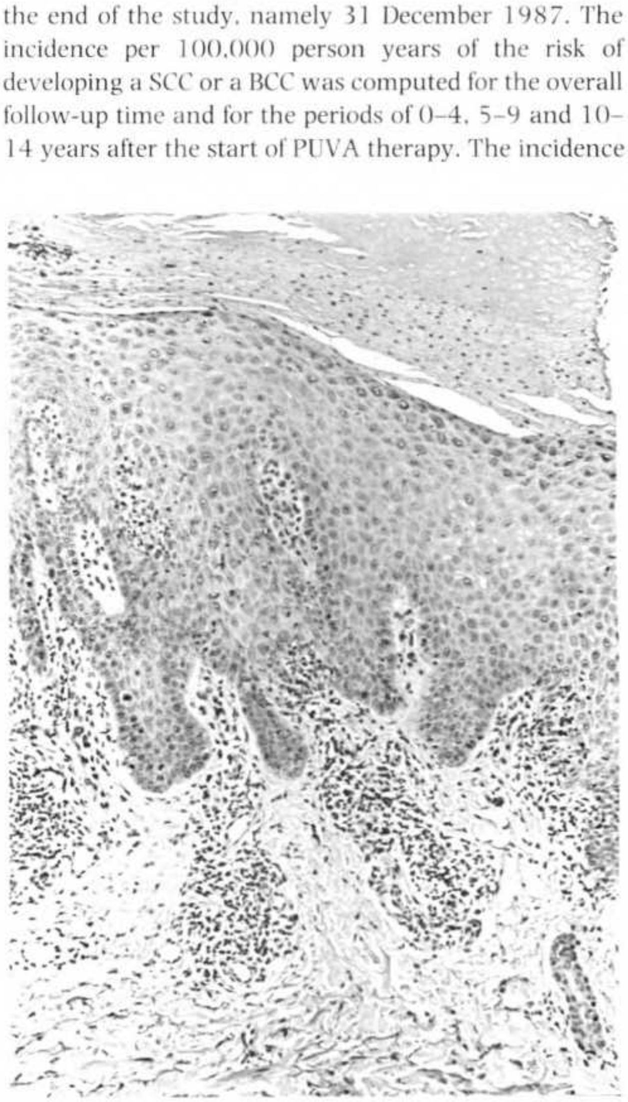

Figure 1. PUVA keratosis: an acanthotic epidermis with hyper- and parakeratosis and a mild perivascular inflammatory infiltrate (haematoxylin and eosin, × 100).

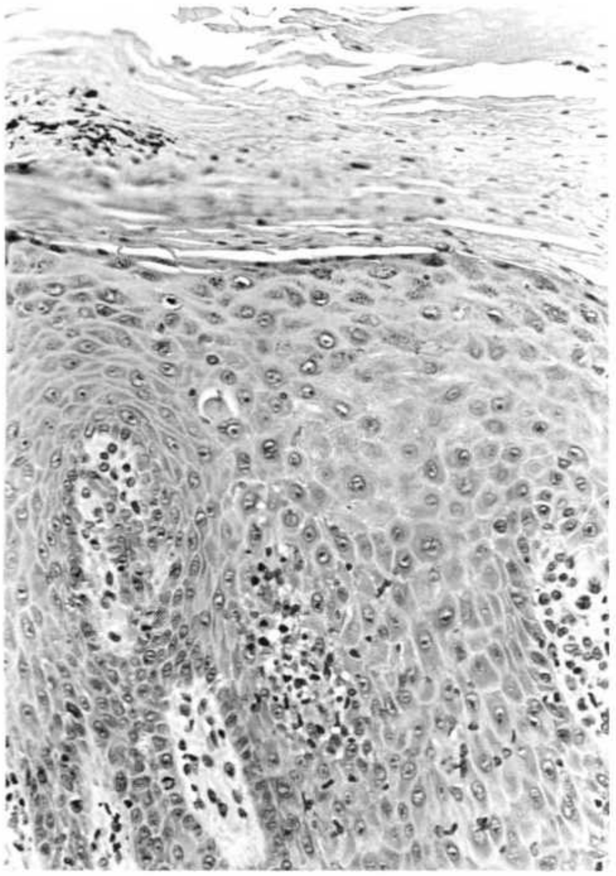

Figure 2. Detail of PUVA keratosis: inflammatory infiltrate slightly invading the epidermis. Apoptotic cell in the spinous-cell layer (haematoxylin and eosin, × 250).

of both SCC and BCC in the group studied was compared with the incidence in the general population by calculating the standard morbidity ratio (SMR).[20] The expected number of skin cancers was calculated by the standard method of applying the age- and sex-specific incidence rates, experienced by the general population, to the number of person years at risk in the group studied. Statistical data for skin cancer were used from the Dutch Eindhoven Cancer Registration (IKZ/SOOZ. Data from 1983–1985. Verhagen-Teulings, personal communication), which region has a similar geographical latitude to Leiden. The expected numbers per 5-year age periods were added together and the totals compared with the observed numbers by means of their ratio (SMR = observed divided by expected numbers).

Survival analysis was used to investigate whether the development of SCC and BCC was affected by various factors; i.e. age, skin type, total UVA dose, number of treatments, history of skin cancer and other risk factors. Each was tested separately with the generalized Wilcoxon rank test. Combinations of these factors were analysed with the Proportional Hazards Model (Cox regression model), which was mainly done to correct for age.

Results

Malignant skin tumours

In our study group of 260 patients with psoriasis we found a total of 37 malignant skin tumours in 11 patients, 13 SCC and 24 BCC. No melanomas were found. In one patient the first tumour to develop was a SCC and in seven patients it was a BCC; in three patients a SCC and BCC were diagnosed at the same time. All the patients with a malignant skin tumour and their relevant details are listed in Table 2. All the tumours were found in patients with skin type III, with the exception of Patients 4 and 7, who both had skin type II.

The incidence of SCC and BCC per 100,000 person years is shown in Tables 3 and 4. The overall incidence of SCC was 210 and 118 for male and female patients, respectively. The incidence of SCC in men increased from 126 after 0–4 years to 1141 after 10–14 years after the start of PUVA therapy. The overall incidence of BCC was 644 for male and 239 for female patients per 100,000 person years. The ratio of the numbers of SCC and BCC that were observed and expected, the SMR, is also shown in Tables 3 and 4. The overall risk for SCC was 12 times higher than that expected in the general population. For BCC there was a fivefold increase in risk.

The odds-ratio of SCC versus BCC was 1 : 8 in the general Dutch population,[21] whereas it was 1 : 2·5 in our study group. The localization of SCC in our patients was 3 : 1 on non-sun-exposed skin versus sun-exposed skin. The distribution of BCC was 5 : 6 on non-sun-exposed areas versus sun-exposed areas. The average time period between the start of PUVA therapy and the diagnosis of the first malignant skin tumour was 6·0 years (standard error of the mean (SEM) 2·8 years) for SCC and 4·7 years (SEM 2·8 years) for BCC.

The correlations between the incidence of malignant skin tumours and the age and skin type of the patients, the total UVA dosage, the total number of treatments, a history of skin cancer, exposure to X-rays, arsenicals, MTX, etretinate and UVB are shown in Table 5. When the statistical test without age correction was used, a relationship was observed between the development of malignant skin tumours and age, cumulative UVA dosage, cumulative number of treatments, a history of skin cancer, arsenicals, MTX and exposure to X-rays. No

Table 2. Details of patients with malignant skin tumours and PUVA therapy

Patient number	Sex	Age	Number of treatments	Total UVA dosage (J/cm²)	Type of tumour	Number of tumours	Location		Years after starting PUVA	UVB	Etretinate	Risk factors
							SE	NSE				
1	M	62	128	205	BCC	1	1	—	6	+	+	MTX
		64	150	226	BCC	3	3	—	8–9			
2	M	62	113	1333	BCC	10	1	9	4–10	—	+	AS, MTX, BC
3	M	77	125	2025	SCC	3	2	1	4–6	+	—	AS
		80	125	2025	BCC	1	—	1	6			
4	F	69	156	1080	SCC	2	—	2	8	—	+	—
5	M	67	30	67	BCC	1	1	—	1	—	—	—
6	M	69	157	1041	BCC	1	1	—	7	+	+	—
7	M	62	70	227	BCC	2	1	1	4–6	+	—	—
8	M	57	171	1725	SCC	2	—	2	9–10	—	+	AS, MTX
		58	171	1725	BCC	1	—	1	10			
9	M	62	187	2282	SCC	6	1	5	3–9	—	+	—
			187	2282	BCC	2	2	—	4–6			
10	F	66	84	1022	BCC	1	1	—	3	+	—	AS
11	F	47	54	270	BCC	1	—	1	1	—	—	—

SE, sun-exposed skin; NSE, non-sun-exposed skin; MTX, use of methotrexate, BC, basal cell carcinoma before start of PUVA treatment; AS, use of arsenicals.

such relationship was observed for skin type nor the use of etretinate and UVB. In the Cox regression analysis (in which a correction for age is made) a relationship was observed between the development of SCC and the total UVA dosage, age at the start of PUVA treatment and a history of arsenic use. No relationship existed between dosage and the development of BCC, but a relationship did exist for age and previous use of arsenicals. Cox regression analysis was also applied using the total number of tumours ($n=37$) but this did not yield any extra information.

Table 3. Incidence of squamous cell carcinoma per 100,000 person years, 'Standardized Morbidity Ratio' (SMR), 95% confidence interval (CI) and *P*-values in male and female psoriatic patients after PUVA therapy

	Years after start of PUVA therapy			
	0–4	5–9	10–14	0–14
Male incidence	126	182	1141	210
SMR	7	8	45	11
95% CI	(0–30)	(0–33)	(0–181)	(2–27)
P (poisson) 1-sided	0·126	0·116	0·022	0·003
Female incidence	0	0	1911	118
SMR	0	0	296	23
95% CI			(0–118)	(0–93)
P (poisson) 1-sided	1·000	1·000	0·003	0·042
Total SMR				12
95% CI				(3–28)
P (poisson) 1-sided				0·000

Benign skin tumours

In 18 patients we found 30 actinic keratoses, while 13

Table 4. Incidence of basal cell carcinoma per 100,000 person years, 'Standardized Morbidity Ratio' (SMR), 95% confidence interval (CI) in male and female psoriatic patients after PUVA therapy

	Years after start of PUVA therapy			
	0–4	5–9	10–14	0–14
Male incidence	641	564	1179	644
SMR	6	4	8	5
95% CI	(2–13)	(1–10)	(0–33)	(2–10)
P (poisson) 1-sided	0·002	0·037	0·112	0·000
Female incidence	221	302	0	239
SMR	4	5	0	4
95% CI	(0–17)	(0–18)		(0–12)
P (poisson) 1-sided	0·210	0·196	1·000	0·087
Total SMR				5
95% CI				(3–9)
P (poisson) 1-sided				0·000

Table 5. Correlation between the development of malignant skin tumours and the listed items, according to the Wilcoxon rank test and the Cox regression model (statistical significance when $P<0{\cdot}05$)

	Wilcoxon rank test *P*-value	Cox regression model *P*-value
	SCC+BCC* ($n=11$)	Total number SCC+BCC* ($n=11$)
Total UVA dosage	<0·05	<0·05 (SCC) NS (BCC)
Total number of treatments	<0·05	NS
Age	<0·05	<0·0005
Skin type	NS	NS
Arsenical	<0·05	<0·05
Methotrexate	<0·05	NS
BCC<PUVA	<0·05	NS
X-rays	<0·05	NS
UVB	NS	NS
Etretinate	NS	NS

NS, not significant.

* Only the first tumour diagnosed in each patient was used for stastistical analysis

patients had 18 PUVA keratoses and one patient a keratoacanthoma. The localization of the actinic keratoses was 14:5 on sun-exposed versus non-sun-exposed areas of the skin and for the PUVA keratoses it was 7 versus 11. In the Cox regression analysis a relationship was observed between the development of these benign skin tumours and the age, skin type, a history of skin cancer and the use of arsenicals.

PUVA freckles

In 70 of the 260 patients (27%) we observed PUVA freckles on areas of the skin not normally exposed to sunlight. No relationship was observed between the development of PUVA freckles and skin type or age. The percentages of patients with PUVA freckles with skin type II, III, IV and V were 29, 26, 38 and 22%, respectively. A positive correlation was observed for the cumulative UVA dosage as well as the cumulative number of treatments.

Discussion

This one-centre, long-term follow-up study of 260 patients with psoriasis treated with PUVA revealed 11 patients with a total of 37 malignant skin tumours and 28 patients with 49 benign skin tumours. No cutaneous melanomas were diagnosed. PUVA-induced lentigines or 'PUVA freckles' were recorded in 27% of the patients in areas of skin not normally sun exposed. The age-adjusted risk of SCC was found to be increased 12 times and that of BCC five times as compared to the risk in the general Dutch population. Using a statistical analysis in which a correction for age was made, a relationship was observed between the development of SCC and the total UVA dosage, the age at the start of PUVA treatment and a past history of the use of arsenicals. The development of BCC was related to age and the previous use of arsenicals.

The psoriasis patients were treated according to the 'aggressive' European schedule, which provides for fewer but higher single-dose treatments as compared to the schedule commonly used in the U.S.A. The average total dose of UVA in our study was 824 J/cm^2, with 67·3% of the patients having received less than 1000 J/cm^2, whereas the average total UVA dose in the American PUVA 48 co-operative study was 1861 J/cm^2.[9]

The ratio of SCC to BCC changed from 1:8 in the general Dutch population to 1:2·5 in our study group. This change is in agreement with previous reports.[2,6]

Although the great majority of SCC and BCC in the general population is localized on the face and neck, we found that three out of four SCC and about half of the BCC were localized on non-sun-exposed skin areas. In PUVA-treated patients we therefore found a trend towards an increased occurrence of skin tumours on non-sun-exposed skin as compared to the general population, although a predilection for occurrence on sun-exposed skin still existed. These findings are suggestive of a primary carcinogenic effect of PUVA treatment, although it must be kept in mind that psoriasis patients are usually frequent sunbathers and expose as much skin as possible to the sun.

We observed that the average period between the start of PUVA treatment and the diagnosis of the first malignant tumour was 72 months for SCC and 57 months for BCC, indicating that an increase of tumour incidence can only be concluded from a follow-up period of at least 6 years. In our study the average follow-up period was 104 months, the longest follow-up period reported to date.

A comprehensive study of the literature on long-term follow-up of patients on PUVA treatment has recently been reported.[9] When we compare our results with the most important and most recent American and European studies one striking difference can be observed.

Although we have used the high single-dose European PUVA schedule and only 18·5% of our patients had risk factors, we found a statistically significant dose-related increase of the incidence of SCC. This has not previously been reported in earlier European studies in which the increase in tumour incidence has been attributed to the high percentage of patients with risk factors.[7,8,11] In American studies, with average cumulative UVA dosages of more than 1500 J/cm^2, this dose-dependent relationship has been reported several times.[5,6,9] A possible explanation of this effect in our study group might be the combination of a longer follow-up period and the fact that these observations have been done through the years in a one-centre setting. Using the Cox regression model, we found neither a positive correlation nor even a trend between the total number of PUVA treatments and the development of skin cancer, which is in accordance with the European reports, and in contrast to the American report of Stern *et al.*[6] Although our study group was smaller than the American group, this finding seems even more suggestive of a major influence of the cumulative UVA dosage on skin tumour development. In clinical practice the European schedule seems to result in a lesser total UVA dosage, although the UVA dose per single treatment is higher in this schedule.

We studied correlations between the development of skin cancer and several risk factors. Carcinogenic risk factors have been reported to be the previous use of arsenicals and MTX, exposure to X-rays and a diagnosis of skin cancer prior to PUVA treatment.[1,2,4–6,8,9,11] We found these factors to be related to the development of malignant skin tumours in our study, using the Wilcoxon rank test, which does not correct for age. Also the cumulative UVA dosage and the total number of PUVA treatments appeared to be related to tumour risk using this test. Treatment with UVB and the use of etretinate did not show a correlation, nor did the skin type reach statistical significance in the malignant tumour group.

When we consider age as a major risk factor (Table 5) and correct for this factor in the Cox regression analysis, it appears that of the risk factors studied, only a prior treatment with arsenicals can be maintained as an additional risk factor apart from the cumulative UVA dosage and the age at the start of PUVA therapy. Neither X-ray therapy, nor the use of MTX were found to be important additional risk factors in our study. Thus, the observed correlations in column 1 of Table 5 seem to be significantly influenced by age-related factors. The occurrence of a BCC prior to PUVA treatment tended to give an additional risk (column 3), although it should be noted that only two patients in our study had one or more BCC prior to PUVA therapy. In a previous study[8] MTX was found to be a risk factor. However, of 97 patients who used MTX, only three patients had MTX as a single risk factor. In our study, 24 of 33 patients had MTX as a single risk factor.

In our study group we found 49 cases of benign tumours of which 18 were of a clinical and histopathological entity which we called 'PUVA keratosis'. These PUVA keratoses were localized on non-sun-exposed skin in 11 out of 18 cases, suggesting a primary relationship between the development of these lesions and PUVA treatment. It is not clear whether we are dealing with a premalignant lesion, although it shows a histological resemblance to the usually highly differentiated SCC as found in PUVA-treated patients.

Although we have reported several long-term adverse effects of PUVA treatment, we would like to stress the great benefit of this therapy for patients with severe psoriasis.

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Selective cytotoxicity of N-acetyl-4-S-cysteaminylphenol on follicular melanocytes of black mice

MANDY WONG AND KOWICHI JIMBOW

Division of Dermatology and Cutaneous Sciences, University of Alberta, Heritage Medical Research Centre, Edmonton, Alberta, Canada T6G 2S2

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Summary

Previous *in vivo* studies have shown that 4-S-cysteaminylphenol (4-S-CAP) and N-acetyl-4-S-cysteaminylphenol (N-Ac-4-S-CAP) have antimelanoma effects and that N-Ac-4-S-CAP produced a 98% depigmentation of hair follicles of black mice. This study investigated the process of selective melanocytotoxicity by N-Ac-4-S-CAP through light and electron microscopy studies of hair follicles obtained from newborn black mice treated with N-Ac-4-S-CAP. Visible changes in follicular melanocytes were found 4 h after intraperitoneal (i.p.) administration. Clumps of melanin granules and areas of melanocytic nuclear condensation were seen in the hair follicles. On electron microscopy there was progressive destruction of melanocytes with swelling of membranous organelles, nuclear condensation, and vacuolation of the cytoplasm, culminating in completely necrotic cells. None of these changes were demonstrated in the surrounding keratinocytes. N-Ac-4-S-CAP appears to have specific, cytotoxic effects on melanocytes actively producing eumelanin. The drug may not affect precursor or dormant melanocytes which retain the ability to become active, melanin-producing cells.

Melanocytes and melanoma cells are unique in their ability to synthesize melanin, which is formed through the conversion of tyrosine to dopa and then to dopaquinone by tyrosinase (EC 1.14.18.1).[1] In an attempt to develop a rational chemotherapy for the treatment of malignant melanoma, a number of studies have exploited the melanin-synthetic pathway to produce compounds which exhibit selective cytotoxicity to these cells. Several highly reactive and potentially toxic intermediates occur during melanin synthesis.[2] The polyquinoid nature of the melanin polymer may promote oxidation of some compounds, and the reduction of others, and allow coupled oxidation of electron donors with reduction of electron acceptors.[3] Most attempts to exploit this potentially hazardous melanogenic pathway have been based upon dopa and related catecholic compounds. However, their use has been limited, largely due to their instability, poor cytotoxicity toward neoplastic cells *in vivo*, and general toxicity from auto-oxidation.[4]

Phenolic and catecholic compounds are known to depigment the skin[5] and we have synthesized a new group of phenolic compounds by combining phenol with cysteine or cysteamine. The resulting cysteinylphenol (CP), cysteaminylphenol (CAP), and related compounds have been shown, in our previous *in vivo* studies[6,7] to have melanocytotoxicity and antimelanoma effects. 4-S-cysteaminylphenol (4-S-CAP) in particular was shown to have a significant antimelanoma effect.[7] However, we subsequently found in an *in vitro* study that 4-S-CAP could produce a cytotoxic aldehyde through interaction with plasma monoamine oxidase (MAO).[8] Homologues of 4-S-CAP were synthesized and compared for their melanocytotoxic and antimelanoma effects. It was found that N-acetyl-4-S-cysteaminylphenol (N-Ac-4-S-CAP) was the most potent melanocytotoxic agent and had an antimelanoma effect equal to that obtained with 4-S-CAP.[9] In this study, we used the black hair follicles of new-born black mice with active eumelanin synthesis to study the mechanism of selective melanocytotoxicity of N-Ac-4-S-CAP and to compare the micoscopic findings with an earlier study using 4-S-CAP.[6]

Methods

Animals

Breeding pairs of C57BL/6J black mice were obtained from the Jackson Laboratory (Bar Harbor, ME, U.S.A.) and their progeny used in the present study.

Preparation and acute cytotoxicity of N-Ac-4-S-CAP

The method of synthesis of N-Ac-4-S-CAP is a modified

Correspondence: Professor K.Jimbow.

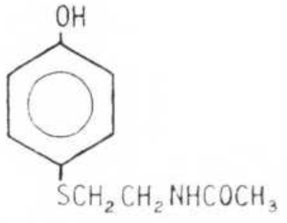

Figure 1. Chemical structure of N-Ac-4-S-CAP.

version of the Wehrmeister reaction,[10] which involves refluxing a mixture of phenol and cysteamine. The chemical structure is shown in Figure 1. The acute cytotoxicity of N-Ac-4-S-CAP was evaluated in a previous study[9,11] using doses from 100 to 1300 mg/kg body weight in mice. The lethal dose of N-Ac-4-S-CAP was found to be 1200 mg/kg.

Method of administration

Experimental group. Thirty newborn C57BL/6J black mice (3-day-old) were injected i.p. with a quantity of N-Ac-4-S-CAP solution, to a total of 300 mg/kg, once a day for 1 or 3 days. Animals were killed for the histological and electron microscopic examination at 4, 12, 16 and 32 h following a single i.p. injection of the compound. Other mice were kept alive to follow the development of white hair follicles.

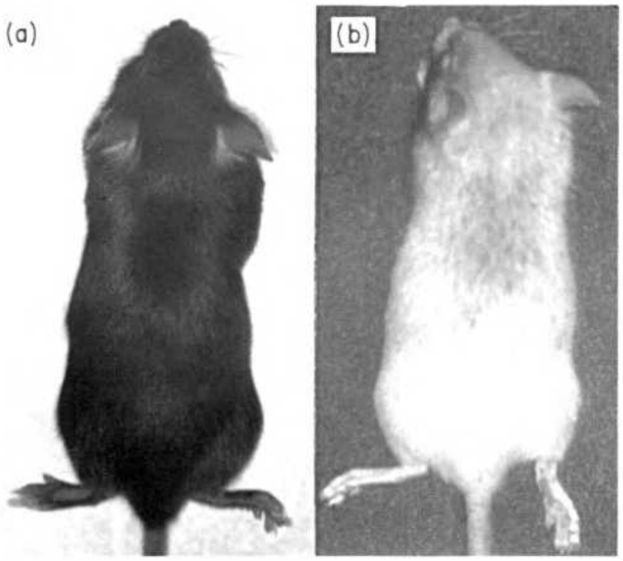

Figure 2. Depigmenting effect of N-Ac-4-S-CAP on new-born black mice (C57BL/6J, 3 weeks old, males). (a) Normal saline: i.p. 0·15 ml for 3 days (at 3, 4 and 5 days of age). (b) N-Ac-4-S-CAP: i.p. 300 mg/kg for 3 days (at 3, 4 and 5 days of age).

Control mice. Twenty new-born C57BL/6J black mice were injected i.p. with normal saline.

Electron microscopy

Skin and hair follicles were biopsied from the mid-dorsum area. Specimens were pre-fixed in Karnovsky's solution, with cacodylate buffer at pH 7·2 and post-fixed with 1% osmium tetroxide in cacodylate buffer. Tissues were stained with 1·5% uranyl acetate in veronal buffer, dehydrated in graded ethanol solutions, embedded in epoxy resin, and sectioned using a Poter–Blum ultramicrotome MT II. For light microscopy, 1-μm-thick sections of the epoxy-embedded specimens were stained with methylene blue solution. Thin sections for electron microscopy were obtained from the same blocks and stained with lead citrate.

Results

Gross appearance of hair follicles after administration of N-Ac-4-S-CAP

Injections of N-Ac-4-S-CAP produced depigmentation of the whole body except for the ear, neck and face where the depigmentation was sparse, in seven of eight new-born mice that had three injections and were left to follow the development of white hair follicles (Fig. 2). One mouse died after the third injection and it was noted that this mouse was born smaller than its siblings. The coats of the mice appeared grey, due to a 1-mm layer of slightly pigmented hair at the top of the hair shaft, but below this the hair was white to the level of the skin. The effect of N-Ac-4-S-CAP was reversible, as the grey coats were replaced with new black coats by 6 weeks of age.

Light and electron microscopy

Control mice. On light microscopy, the follicular melanocytes were located adjacent to the hair papilla of the new-born mouse, in the superior half of the anagen hair follicle (Fig. 3a). The melanocytes contained numerous brown–black melanin pigments which extended into their long dendritic arms that surrounded the neighbouring keratinocytes. No vacuolation was seen in the cytoplasm of these melanocytes and the fibroblasts in the hair papilla were also normal. Electron microscopy revealed many ellipsoidal, stage IV melanosomes and occasional stage III melanosomes in the cytoplasm of the melanocytes (Fig. 4).

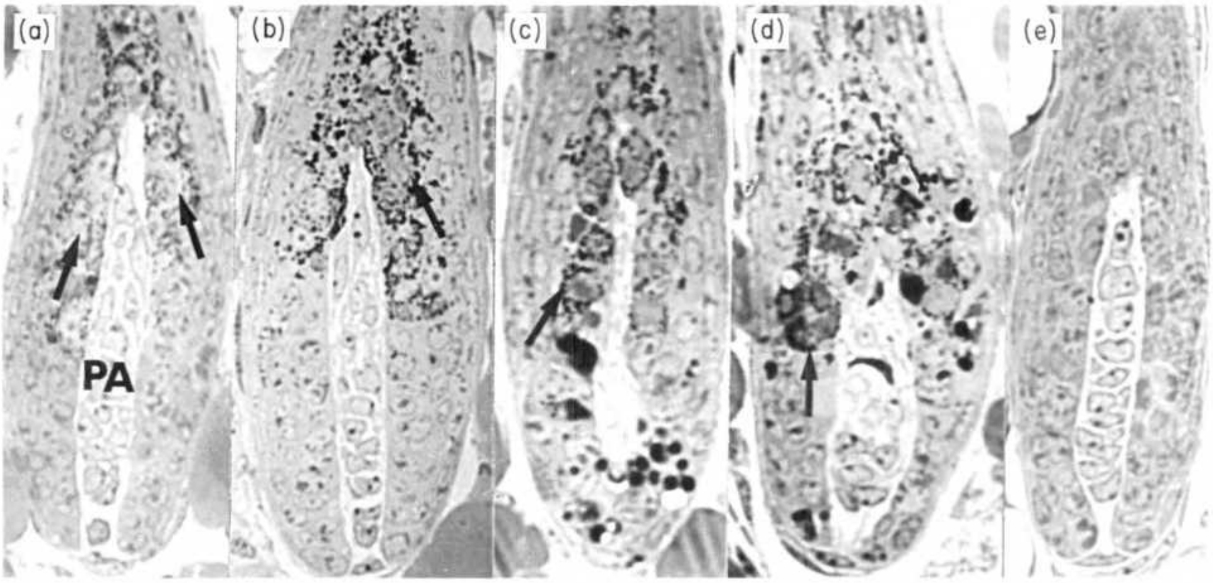

Figure 3. Light microscopic changes in follicular melanocytes after a single i.p. administration of N-Ac-4-S-CAP at 300 mg/kg (C57BL/6J, 3 days old). 1-μm-thick epoxy sections of hair follicles. (a) At 12 h post-injection of normal saline. PA, papilla; arrows, normal melanocytes (× 870). (b) At 4 h post-injection of N-Ac-4-S-CAP. Arrow, darkly staining nucleoplasm of melanocytes (× 870). (c) At 12 h post-injection of N-Ac-4-S-CAP. Arrow, degeneration of melanocytes and the aggregation of pigment granules (× 870). (d) At 16 h post-injection of N-Ac-4-S-CAP. Arrow, degeneration of melanocytes (× 870). (e) At 32 h post-injection. None of the melanocytes are visible (× 870).

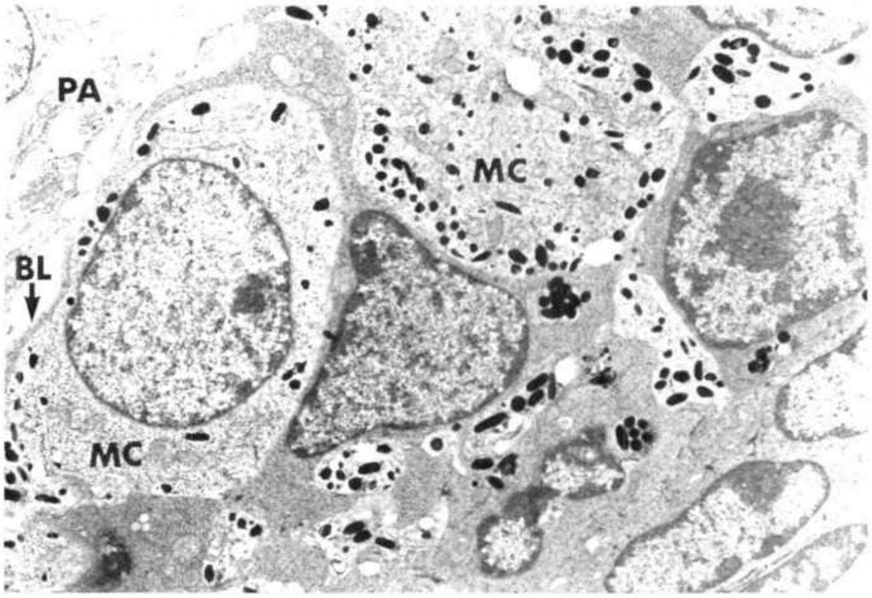

Figure 4. Normal follicular melanocytes in a 3-day-old, C57BL/6J mouse with an i.p. injection of normal saline (0·15 ml). There are two functioning melanocytes, MC, in the hair bulb above the hair papilla, PA. BL, Basal lamina. Dendritic processes are seen in the intercellular spaces of keratinocytes. (× 2560).

Experimental mice. Four hours after injection of N-Ac-4-S-CAP, no obvious degenerative changes were seen on light microscopy (Fig. 3b). However, the nucleoplasm of the melanocytes stained darker than in control hair follicles. The surrounding keratinocytes and fibroblasts remained unchanged. The beginning of degeneration within melanocytes could be clearly seen on electron microscopy with an increased density of the entire nucleoplasm and scattered heterochromatin both in the periphery and in the centre of the nucleus (Figs 5a and b). There was also a diffuse darkening of the entire cytoplasm, with obvious dilatation of the endoplasmic reticulum which contained amorphous material. There was dissociation of the outer membrane of some melanosomes in the cytoplasm of melanocytes. The mitochondria did not show any structural change at this stage.

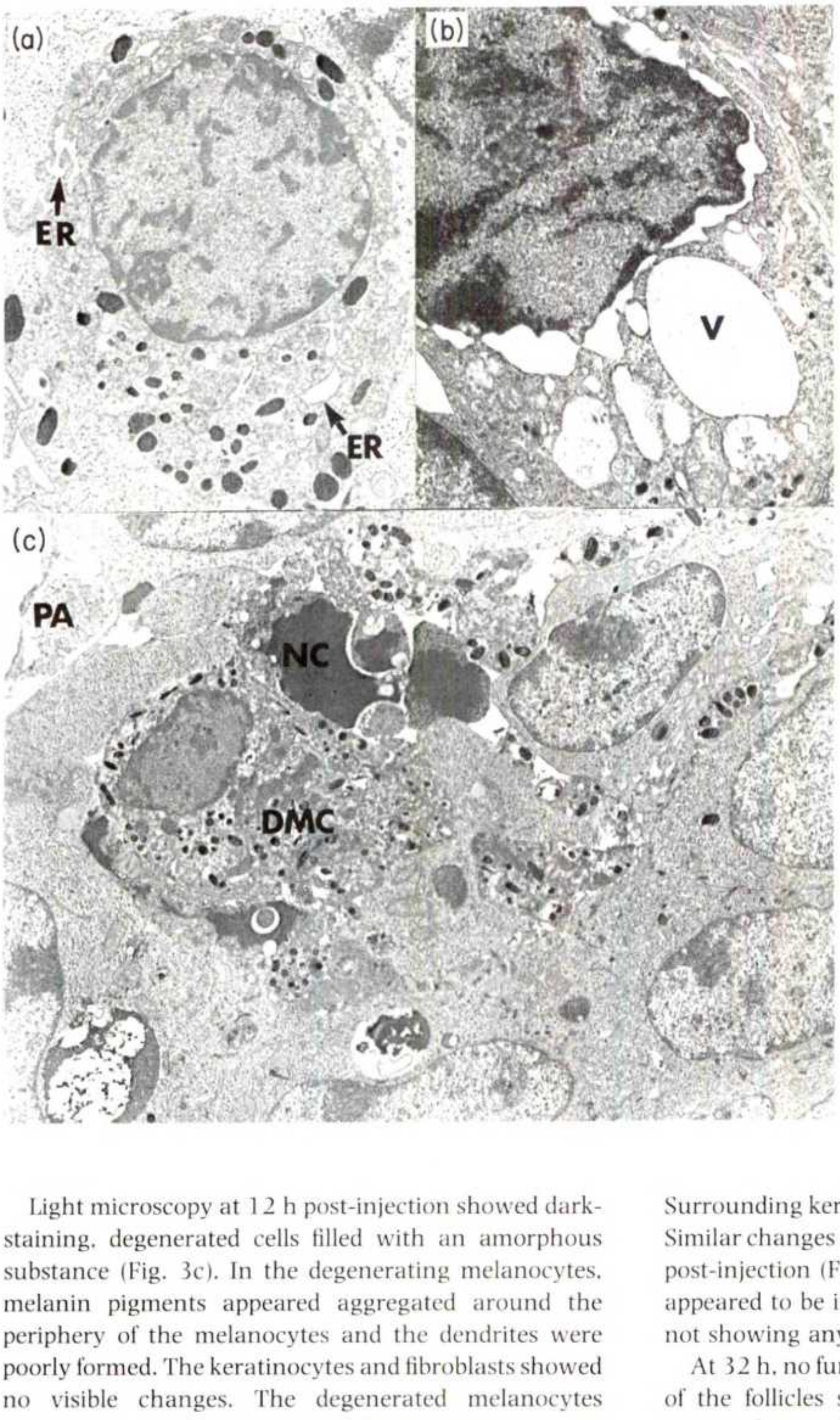

Figure 5. Degenerating melanocytes seen after i.p. administration of N-Ac-4-S-CAP. (a) Low-power view of a melanocyte 4 h post-injection with dilated endoplasmic reticulum, ER (× 7380). (b) High-power view of degenerating follicular melanocytes 4 h post-injection reveals large vacuoles, V, and nuclear condensation (× 14,760). (c) Low-power view of degenerating melanocytes, DMC, 16-h post-injection seen in the hair matrix above the papilla, PA. Some show a complete necrosis, NC (× 7380).

Light microscopy at 12 h post-injection showed dark-staining, degenerated cells filled with an amorphous substance (Fig. 3c). In the degenerating melanocytes, melanin pigments appeared aggregated around the periphery of the melanocytes and the dendrites were poorly formed. The keratinocytes and fibroblasts showed no visible changes. The degenerated melanocytes appeared as a homogeneous, diffusely staining amorphous mass that contained many vacuoles. Degenerated mitochondria were seen at this stage.

At 16 h post-injection, greater numbers of degenerated cells were present, and were very darkly stained with numerous vacuoles. Some degenerating melanocytes contained clumps of melanin pigments (Fig. 3d). Surrounding keratinocytes and fibroblasts were normal. Similar changes were seen on EM to those described 12 h post-injection (Fig. 5c). The surrounding keratinocytes appeared to be ingesting necrotic melanocytes, though not showing any degenerative changes.

At 32 h, no functioning melanocytes were seen in any of the follicles sectioned (Fig. 3e). The keratinocytes appeared normal without melanin pigment.

Discussion

Melanocytes in anagen follicles are actively involved in mitosis, melanogenesis and tyrosinase synthesis. In a previous study, 4-S-CAP was shown to produce a selective cytotoxicity to melanocytes and similar results were found with N-Ac-4-S-CAP. These findings included (i) visible degenerative changes occurring as early as 4 h in melanocytes after a single i.p. injection of N-Ac-4-S-CAP; (ii) degeneration and lysis of melanocytes, manifested by dilatation of membranous organelles and dissociation of the outer nuclear membrane from the nucleoplasm, and completely necrotic cells; (iii) no signs of degeneration in the surrounding keratinocytes and fibroblasts. These indicate a selective cytotoxicity of N-Ac-4-S-CAP to melanocytes, and that the cytopathic reaction is not attributable to the binding of our compound to melanin.

The coat of the drug-injected new-born mice was not the jet black colour of the control mice. The layer of pigmented hair at the tip of the hair shaft, giving the coat a grey colour was due to melanin formed by active melanocytes prior to injection of N-Ac-4-S-CAP at 3 days of age. The depigmenting potency of our compound was evident as the pure white coat of hair beneath the grey top layer. The effect of our drug was reversible, however, as the white coat was completely replaced with a new, black coat in the next hair cycle. This reflects the possibility of a pool of precursor melanocytes present in the hair follicle which are not active and thus are not affected by the compounds. In the next hair generation, some of the cells from this pool may become activated and divide, producing a new set of melanocytes which go on to pigment the hair. The presence of these inactive precursor or dormant melanocytes has been identified by previous electron microscopic studies.[12,13] In addition, certain areas of body surface, i.e. ear, neck and face, showed an incomplete, non-uniform depigmentation. This again may indicate that there are certain melanocytes that do not respond to our compounds.

A major difference in results obtained after injection with 4-S-CAP compared to N-Ac-4-S-CAP was the depigmenting potency of these drugs. In a previous study,[9] N-Ac-4-S-CAP at a dose of 300 mg/kg produced 97·6% depigmentation by a follicular melanocyte assay. 4-S-CAP at the same dosage produced only 86·6% depigmentation. This may be because 4-S-CAP is a substrate for MAO, whereas N-Ac-4-S-CAP is not.[14] Thus, there is increased bioavailability of N-Ac-4-S-CAP to target cells *in vivo* resulting in a greater melanocytotoxic effect. In addition, our earlier study showed that 4-S-CAP could produce a cytotoxic aldehyde through interaction with plasma MAO,[8] which would explain the greater number and severity of side-effects seen in mice injected with 4-S-CAP as compared to N-Ac-4-S-CAP. No remarkable side-effects were noticed after N-Ac-4-S-CAP administration.

The currently accepted mechanism of selective toxicity of phenolic compounds has been previously discussed.[4,6,11,15,16] Both N-Ac-4-S-CAP and 4-S-CAP are good substrates for mammalian tyrosinase and can be incorporated into melanin synthesis in the presence of dopa as a cofactor.[15] Melanin precursors or intermediate metabolites in the melanin synthetic pathway have been shown to possess antimelanoma effects in many *in vitro* and some *in vivo* systems. The cytotoxicity of dopa and related catecholic compounds depend on autoxidation, producing superoxide and hydroxide radicals.[2,4] These compounds are oxidized by tyrosinase to the *o*-quinone forms which then act on sulph-hydryl groups to inhibit DNA polymerase alpha. Similarly, 4-S-CAP and N-Ac-4-S-CAP may become cytotoxic upon tyrosinase mediated oxidation to *o*-quinone forms and conjugation with sulph-hydryl enzymes. The quinones that escape thiol conjugation may polymerize to form melanin-like pigments, possibly generating hydrogen peroxide and other active oxygens.[15]

In this study, electron microscopy showed that dissociation of the melanosomal membrane and dilatation of the endoplasmic reticulum (ER) and Golgi apparatus were among the first degenerative changes seen. These may be explained by considering the morphogenesis of melanosomes and melanization. It is known that tyrosinase and the structural proteins that make up the melanosomal matrix and membrane are both synthesized on the ribosome of rough ER. However, the assembly and maturation of these components occurs in separate areas of the cell. The structural proteins destined to form the stage I melanosomes are assembled in the dilated tips of smooth ER or degranulated rough ER or multivesicular bodies that are connected to rough ER. In contrast, tyrosinase is assembled in a particular area of the Golgi complex (Golgi–endoplasmic reticulum–lysosome system (GERL)) and when matured, is transferred from the GERL to stage I melanosomes by budding off of a dilated tip.[1,17] The coated vesicles are carriers not only of tyrosinase and other post-tyrosinase regulatory factors but also of melanin precursor monomers.[17,18] Thus, tyrosinase-mediated lipid peroxidase due to N-Ac-4-S-CAP metabolites would occur in the membranes of the Golgi, ER, and melanosomes first, with damage to other membranes of the cell later, culminating eventually in necrosis of the entire cell.

In the search for compounds exhibiting antimelanoma effects, several other investigators have performed similar experiments using naturally occurring phenolic precursors isolated from the common mushroom, *Agaricus bisporus*. The parent phenol, gamma-L-glutaminyl-4-hydroxybenzene (GHB), was found to produce reversible depigmentation of the hair of black mice[14] as well as exert an inhibitory effect on the growth of murine B-16 melanoma *in vivo* and *in vitro*. It is believed that mammalian melanoma cells, possessing tyrosinase, oxidize the phenol to an inhibitory quinone.[19] Monohydroxy and dihydroxy analogues of GHB have also been synthesized and studied for their *in vitro* and *in vivo* antimelanoma effect.[20] Similar approaches have been reported by Riley and his associates.[21] 4-Hydroxyanisole was found to be a potent depigmenting agent and to have *in vivo* and *in vitro* antimelanoma effect in animals and humans. To date, it appears that the phenolic derivatives offer the most rational approach to the chemotherapy of malignant melanoma in terms of their selective melanocytotoxic and antimelanoma potential and their limited side-effects. N-Ac-4-S-CAP, upon oxidation by tyrosinase to an *o*-quinone form that exerts a cytotoxic effect, has been shown to possess the most melanocytotoxic and antimelanoma potential of the phenolic melanin precursors. Black hair follicles of mice are involved exclusively in eumelanogenesis, whereas melanoma tissues are usually engaged in both eu- and phaeomelanogenesis.[22] The role of the phaeomelanin synthetic pathway must be considered in the possible mechanism of the antimelanoma effect by N-Ac-4-S-CAP.

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Long-term therapy of HIV-associated Kaposi's sarcoma with recombinant interferon α-2a

F.M.SCHAART, B.BRATZKE, Zb.RUSZCZAK, R.STADLER, G.EHLERS* AND C.E.ORFANOS

*Department of Dermatology, University Medical Center Steglitz, The Free University of Berlin, *Department of Dermatology, City Hospital Britz/Neukolln, Berlin, Germany*

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Summary

Five young male patients with HIV-associated Kaposi's sarcoma (KS) were treated with recombinant interferon α 2a (rIFN-α-2a) over a period of 2–2·5 years. An IFN dose of 18×10^6 IU was given subcutaneously every day during the first 3 months of treatment and then on alternate days. Additional treatment with radiotherapy and laser therapy was given and, in some cases, isolated skin nodules were excised. Within 7 months of initiation of therapy one patient had a complete remission of his tumours, however, tumour progression recurred after the patient discontinued treatment. In another patient the tumour cleared within 9 months of rIFN therapy, and after 52 months he is still free of KS . The condition of a third patient tended to become stabilized during the first 6 months of therapy, but after 60 months there has been a slow progression. The fourth and fifth patients died 25 and 28 months, respectively, after the histological diagnosis of KS and the initiation of treatment. While on therapy with rIFN-α-2a, no life-threatening opportunistic infections occurred. The side-effects were mostly well tolerated, and no severe changes in haematological parameters were caused by the therapy.

The epidemic variant of Kaposi's sarcoma (KS) is a malignant tumour of vascular origin associated with HIV infection. It occurs almost exclusively in homosexual and bisexual males and was first described in 1981.[1,2] According to a recent WHO report,[3] 11·5% of the 34,967 AIDS patients registered in 31 European countries up to 31 March, 1990 showed manifestations of mucocutaneous Kaposi's sarcoma. The current percentage of AIDS patients with manifestations of KS is declining world-wide, but the total number of patients with HIV-associated KS may presently exceed 35,000. HIV infection can now be diagnosed much earlier and opportunistic infections (OIs) in AIDS patents can be more effectively controlled but still an increased number of HIV-infected individuals die of widespread KS. The view that KS occurring in HIV-infected patients has a good prognosis, has long been abandoned.

Although there is no cure for HIV-associated KS, rIFN-α has been shown to be beneficial when given in the early stages of the disease.[4–11] Hence, rIFN-α-2a was recently introduced as a mode of systemic treatment in several European countries and in the U.S.A., although the exact mechanisms of its antitumour action still remain unclear. The high dosage of rIFN-α (18–36 mio/E.s.c.) required to induce tumour regression has led to the suggestion that, besides having known antiviral and immunomodulatory effects, its antitumour activity is based mainly on growth inhibitory effects. Recombinant IFN-α-2a produced a clinical response in about 40–50% of AIDS patients with disseminated mucocutaneous KS.[5,6,11] A reduction in the number of endothelial cell types in KS lesions was detected in histological and electron microscopy studies after treatment with rIFN-α-2a.[12] However, tumour recurrences, rapid tumour progression and/or opportunistic infections occurred after cessation of therapy. We therefore believe that HIV-associated KS must be treated *continuously* over a long period of time (if not permanently). This paper reports our experiences with five patients who had continuous rIFN-α-2a treatment over a period of more than 2 years.

Methods

Patients

Between October 1982 and June 1990, a total of 76 patients with histologically confirmed HIV-associated KS

Correspondence: Dr Frank-Matthias Schaart, Department of Dermatology, University Medical Center Steglitz, The Free University of Berlin, Hindenburgdamm 30, D-1000 Berlin 45, Germany.

were seen in our dermatological department in Berlin. Five of these patients underwent continuous treatment with rIFN-α-2a for a period of more than 2 years (24–29 months). All the patients were young homosexual or bisexual males. Their mean age at the start of therapy was 29 years (range 23–44 years). They were all HIV-positive in ELISA and Western blot tests and all showed disseminated mucocutaneous lesions typical of KS. The T-helper/T-suppressor ratio before therapy varied from 0·55 to 1·33 (median = 0·83) and T-helper counts ranged from 260 to 881/mm^3 (median = 580/mm^3). None of these patients had a history of severe opportunistic infection before the diagnosis of HIV-associated KS was made.

Continuous rIFN-α-2a therapy schedule

After histological confirmation of KS, the patients received an initial daily subcutaneous dose of 18×10^6 IU rIFN-α-2a for 3 months after which they received a dose every other day. In the initial phase of therapy, the patients were allowed to stay in hospital until they had become familiar with the side-effects of the drug and the injection procedure (1–3 weeks). Clinical examinations and laboratory tests were carried out at regular intervals. Additional, radiotherapy, laser therapy and/or excision of isolated cutaneous nodes and nodules was performed during the 2-year period.

Results

Clinical observations

Patient 1. This patient aged 44 years at the start of therapy first presented in May 1987 with livid, partly ulcerated tumourous lesions on the right foot and arm (Fig. 1a). These lesions had arisen and grown to the present size within 2 months. The T-helper cell count was 612/mm^3. The continuous rIFN-α-2a therapy schedule was initiated, and within 7 months complete regression (100%) of KS lesions was observed leaving blue–brownish areas of hyperpigmentation that were histologically tumour-free (Fig. 1b). However, therapy was continued for another 11 months. Three months after cessation of therapy, against our advice, new KS lesions occurred on the same leg. rIFN-α-2a therapy produced a slow regression of these lesions and the patient is now, 37 months after histological diagnosis of KS, on treatment with vincristine 2 mg intravenously and bleomycin 15 mg intravenously every other week because of new tumour progression on the right inner ankle. During the long-term rIFN-α-2a treatment, Patient 1 had an episode of severe frebrile tonsillitis and also developed oral candidiasis, which was successfully managed with oral antifungals.

Patient 2. This patient, aged 29 years, was diagnosed as HIV-positive in the summer of 1985 and in February 1986 presented to our department with three lesions of KS on the legs. The pretreatment T-helper cell count was 260/mm^3. Continuous therapy with rIFN-α-2a was started and within 12 months complete remission of the skin tumours was observed. Patient 2 also decided to discontinue therapy after 24 months but to date after 52 months there has been no recurrence of the KS lesions. During the course of treatment the patient had a number of infections and oral hairy leucoplakia was diagnosed in March 1989. The patient has had psoriasis since childhood, but this has remained unaltered during therapy.

Patient 3. This patient, aged 23 years, was diagnosed as HIV-positive in February 1985. The clinical and histological diagnosis of diseminated KS was established in June 1985 with four livid nodules on the trunk and left leg. The pretreatment T-helper cell count was 881/mm^3. Continuous rIFN-α-2a therapy was initiated, and stabilization of HIV-associated KS was noted in the first 6 months. Slow progression of the disease was observed from the seventh month of therapy but could be controlled by additional therapy (i.e. radiation, excision). The drug was administered until November 1987 (29 months), including two arbitrary interruptions of 1 and 4 months, respectively, until the patient finally decided to discontinue treatment. Because of accelerated progression of KS lesions 1 month later, we started the patient on a modified therapy plan comprising zidovudine 1000 mg p.o. in combination with rIFN-α-2a (18×10^6 IU s.c. every 2 days). Partial tumour regression (> 50%) was again achieved within 3 months, but, after withdrawal from therapy by the patient, rapid progression of new tumours occurred in November 1988. The reintroduction of combined rIFN-α-2a/zidovudine therapy was unsuccessful in controlling the disease. Commencing in March 1989, the patient then received either 2 mg vincristine or 0·7 mg vinblastine/kg body weight intravenously, alternating every week for 8 months. Therapy with rIFN-α-2a was reintroduced, combined with 2 mg intravenous vincristine every other week. The lesions of KS remained widespread. The patient is still able to actively participate in daily life (60 months). The following HIV-associated

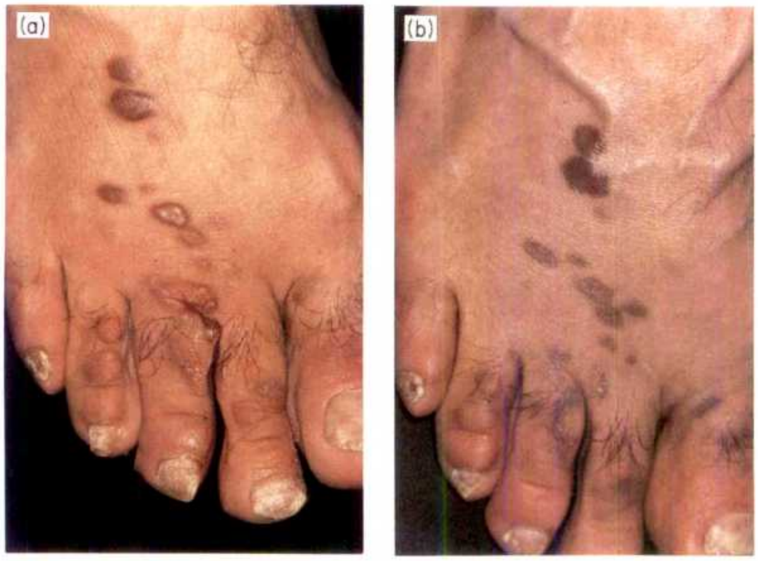

Figure 1. Patient 1. (a) Partly ulcerated tumour on the right foot before therapy; and (b) 6 months after exclusive rIFN-α-2a therapy.

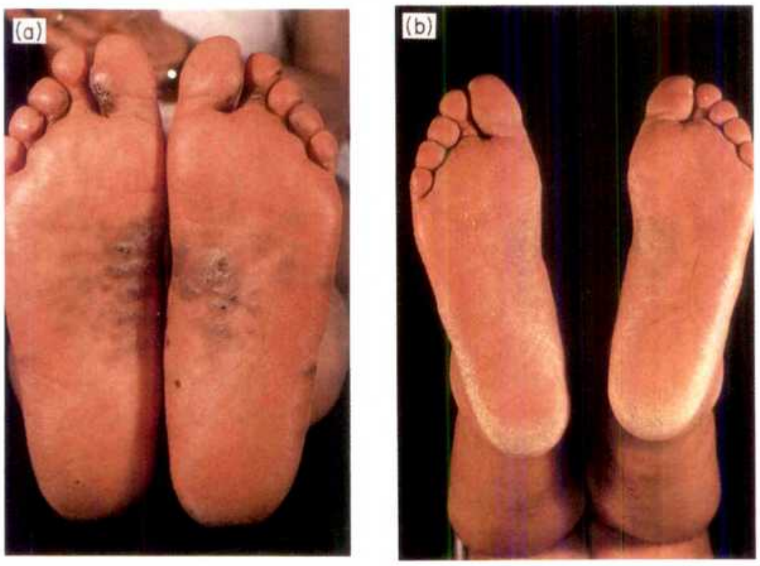

Figure 2. Patient 5. (a) Kaposi's sarcoma before therapy and (b) 3 months after exclusive rIFN-α-2a therapy.

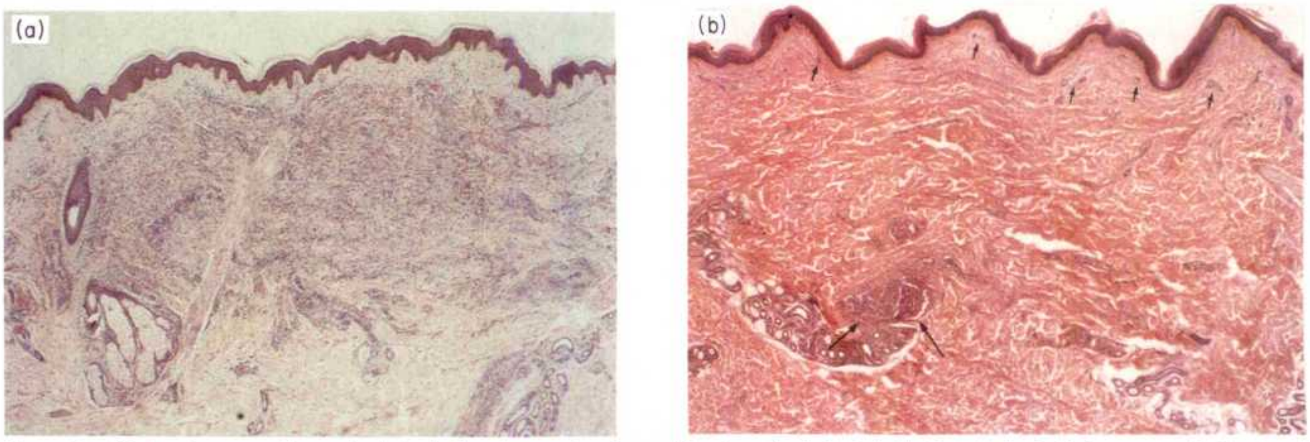

Figure 3. (a) Light microscopy of a papular lesion before therapy showing proliferation of endothelial cells. Note the lack of inflammatory infiltrate and only discrete extravasation of erythrocytes. (b) Eight months after exclusive rIFN-α-2a therapy: absence of proliferating endothelial cell aggregates; a few vessels with cuboidal endothelial cells are present in superficial dermis (arrows); residual KS in the deep dermis surrounding sweat glands (long arrows) (haematoxylin and eosin ×40).

diseases were diagnosed during the course of rIFN-α-2a treatment: necrotizing zoster, oral and intestinal candidiasis, acute bronchitis, inguinal erysipelas, perianal abscesses, perianal condylomata acuminata, disseminated folliculitis and two acute psychotic episodes, which were regarded as an organic psychosyndrome accompanying HIV infection.

Patient 4. This patient was 24 years old when diagnosed as being HIV-positive in August 1985 following the development of segmental herpes zoster. The first KS lesions appeared in December 1985 and were confirmed histologically in February 1986. Several KS lesions were on the trunk, extremities and face. The T-helper cell count was 580/mm^3. Continuous rIFN-α-2a therapy was initiated, and within 6 months there was a partial regression of the lesions. Against our advice the patient failed to take the drug for a period of 3 months, which led to renewed tumour progression. In May 1987, continuous rIFN-α-2a therapy was reinitiated, and the disease was successfully stabilized until May 1988, when rapid tumour progression occurred on his face, trunk and extremities. The patient died in July 1988, 27 months after the initiation of therapy. The survival time calculated after initial histological diagnosis of KS was 28 months.

Patient 5. This patient, aged 42 years, was diagnosed as HIV-positive in July 1985. He presented in June 1986 with partially ulcerated KS lesions which were around the genitals, on both feet (Fig. 2a) and in the mouth. The T-helper cell count was 574 cells/mm^3. Continuous rIFN-α-2a treatment was commenced and 3 months later there was partial regression of the lesions which lasted more than 12 months (Fig. 2b). However, the disease progressed and slowly disseminated. After 26 months treatment with rIFN-α-2a was replaced with cytostatic drugs (2 mg vincristine i.v. or 0·2 mg vinblastine i.v. per kg body weight, alternating every week). These had to be discontinued because of neurological side-effects in January 1988. An explosive tumour progression occurred, and rIFN-α-2a therapy (18×10^6 IU s.c./die) was reintroduced, leading to temporary stabilization but rapid progression of the tumours on large areas of the face, oral mucosa, trunk and lower extremities, resulted in the death of the patient 25 months after the histological diagnosis of KS. Accompanying diseases observed during systemic rIFN-α treatment were: herpes zoster, intestinal parasitosis, oral and intestinal candidiasis and hairy leucoplakia.

Histological examinations

All the patients had mainly papular and/or nodular cutaneous lesions which showed Kaposi's angiosarcoma in various stages (Fig. 3a). Atypical endothelial cells with numerous Weibel–Palade bodies were seen on electron microscopy and they were accompanied by only slight proliferation of pericyte-like cells (Fig. 4). Thin-walled capillaries and blood lakes and other features characteristic of haemorrhagic KS lesions were almost absent in the skin nodules.

After long-term rIFN-α-2a treatment, complete or partial remission of KS-lesions were confirmed histologi-

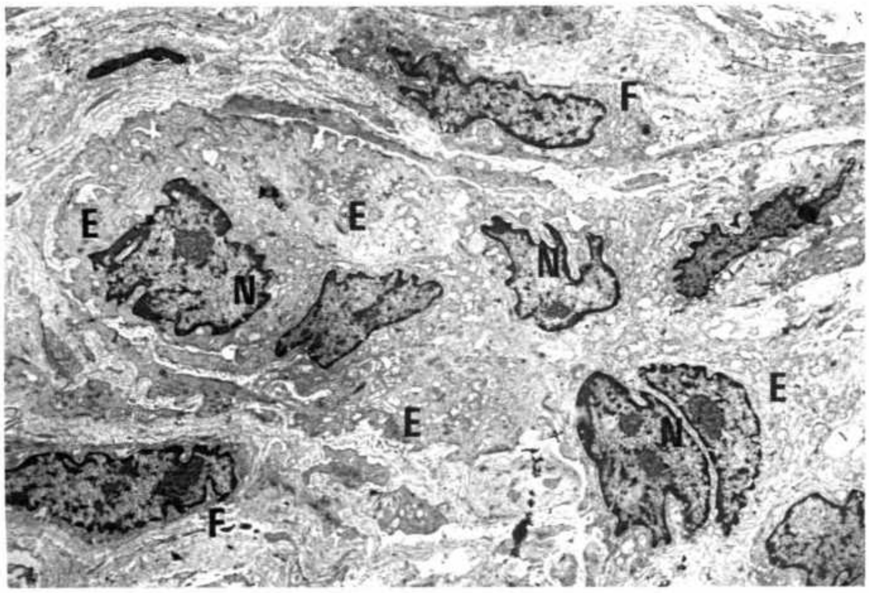

Figure 4. Ultrastructure of a papular KS skin lesion: proliferation of atypical endothelial cells (E) is observed, forming occluded vascular-like structures; very active, multilobular nuclei (N) with multiple nucleoli and active nuclear organizing centres. F, fibroblastoid cells (with signs of proliferation similar to endothelial cells) (uranyl acetate/lead citrate × 3200).

cally. In particular, there was a dramatic decrease or a complete absence of endothelial cell aggregates after 6–8 months of continuous rIFN-α-2a therapy. In some cases, endothelial aggregates were then found only in the periadnexial dermis (Fig. 3b), and in some areas the cells showed degenerative features. At the ultrastructural level, they demonstrated irregular nuclei, their mitochondria were swollen and partially destroyed, and the endoplasmic reticulum was enlarged and filled with electron-dense granular material. In the cytoplasm of these cells undergoing degeneration, erythrocyte fragments were seen. Erythrocyte extravasates and a large number of macrophages with signs of intense phagocytosis were found along with some mast cells that contained irregular and incomplete granules.

Side-effects

Laboratory parameters. A transient reduction in the white blood cell counts (WBC) was seen in the first 6 months of therapy in all the patients (Fig. 5a). The lowest values were observed in weeks 2–8. The WBC subsequently became stabilized with low normal or slightly leucopenic levels. In three patients, the platelet counts were initially reduced, but became stabilized in 3·5–5 months. The platelet counts in the remaining two patients (Patients 3 and 4) increased during the course of rIFN-α-2a therapy. No significant changes in the red blood count, haemoglobin content, serum creatinine or transaminases were observed during therapy. Continuous rIFN-α-2a therapy caused no significant increase in either the T-helper/T-suppressor ratio (Fig. 5b) or the T-helper count (Fig. 5c).

Subjective symptoms. Four of the five patients complained of flu-like symptoms including fever (sometimes with chills), night sweat, headaches, general weakness, fatigue and myalgia, arising 2–12 h after the injection. These symptoms became less troublesome as the treatment progressed.

Loss of appetite was mentioned by three patients, sleep disorders by two, and diffuse alopecia by one, probably as a result of the therapy. One patient had no subjective side-effects. In general, continuous administration of rIFN-α-2a was well tolerated, and discontinuation of the treatment due to changes in laboratory parameters or due to severe subjective side-effects was normally not necessary.

Discussion

Since the first description of interferon by Isaacs and Lindenmann in 1957,[13] the important role of this group of cytokines in cell biology has been documented by a large number of studies. By means of gene technology, large amounts of recombinant interferons can be easily obtained. IFN-α-2a produced by transforming *Escherichia coli* bacteria, has been shown to be clinically

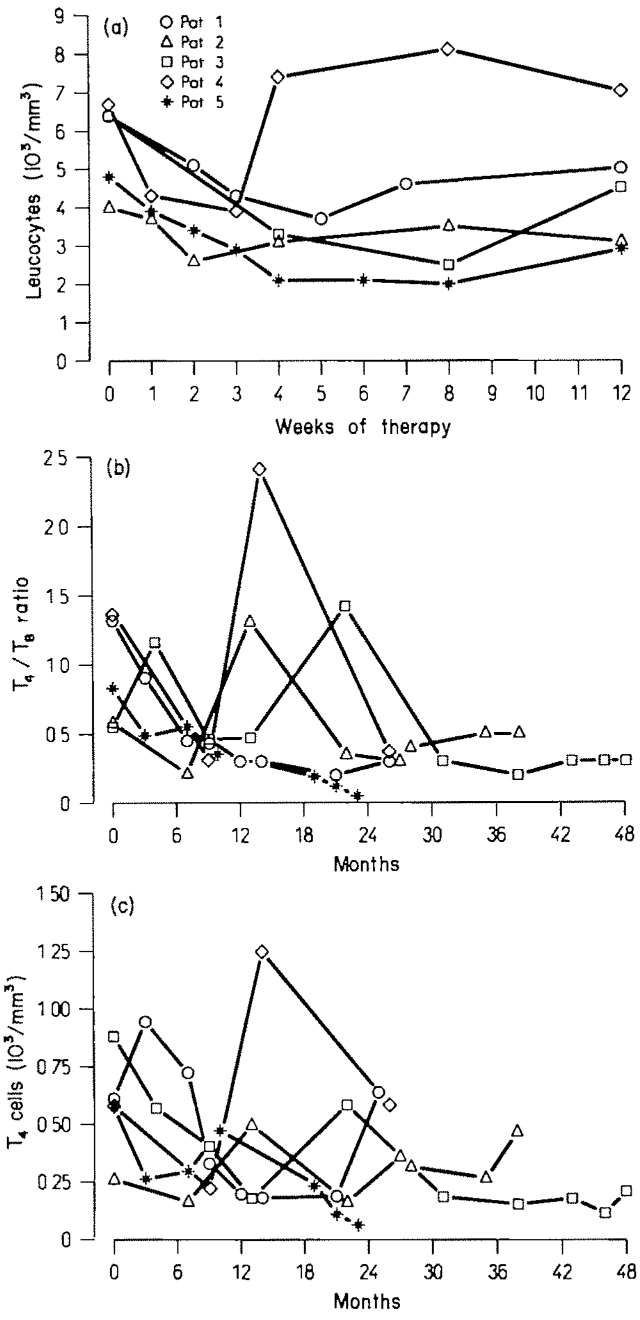

Figure 5. Changes in laboratory investigations during therapy. (a) Lowering of white blood count at weeks 2–8 after start of therapy followed by subsequent stabilization at low normal or slightly leucopenic levels. (b) No significant increase of T-helper/T-suppressor ratio or (c) T-helper count was observed.

effective in common warts, condylomata acuminata, Behçet's disease, cutaneous T-cell lymphoma and in HIV-associated Kaposi's sarcoma.[14] Results of long-term rIFN-α-2a therapy in the five patients with disseminated mucocutaneous KS described here confirm and supplement our previously published findings.[4]

Two patients showed complete remission of KS lesions within 7–12 months of therapy, but the disease recurred in one of the patients after withdrawal of therapy. The other patient, who has been treated with rIFN-α-2a for 24 months, is still free of KS lesions today (52 months after histological diagnosis of KS). In one of our patients, marked regression and long-lasting stabilization of the disease was achieved by rIFN-α-2a therapy. Now, 60 months after the histological diagnosis of Kaposi's sarcoma, the patient is still able to actively participate in daily life.

These observations show the necessity of continuous treatment with rIFN-α-2a for as long as the drug is tolerated. When tumour progression occurred after cessation of therapy, renewed administration of rIFN-α-2a was potent enough to induce marked tumour regression or stabilization of the disease, as we observed in three of our patients. However, complete tumour regression could not be achieved. Two patients died 25 and 29 months after histological diagnosis of KS. Our results show a significant increase in survival time as compared with patients who received no treatment for HIV-associated KS lesions (3–7 months).[11]

Both clinically and morphologically confirmed IFN-α-2a-dependent regression of KS-lesions has been seen, particularly in maculopapular and nodular lesions. In patients with flat, haemorrhagic macules, such long-term effects of rIFN-α-2a therapy were not observed. This is in accordance with our previous findings where only a partial influence of the drug on proliferating KS-related cell populations was observed in electron microscopic studies.[12]

Ultrastructural findings described in this paper suggest cytotoxic effects of rIFN-α-2a on proliferating lesional cells expressing endothelial markers. Interestingly, rIFN-α-2a was recently found to have antiproliferative and cytotoxic effects in human dermal microvascular endothelial cells *in vitro*.[15] It was reported that the antiproliferative effects of rIFN-α-2a are dose-dependent and reversible, and can be achieved only in the continuous presence of the drug in the culture media. This may partially explain why disease may recur in patients who withdraw from or interrupt therapy.

Only one patient had a T-helper count of less than 400/m^3 before the initiation of therapy. However, he was the only patient who achieved complete remission from the disease, and he is still disease-free today. Obviously, a well-preserved immune system is important, but it does not seem to be the key to predicting effectiveness of rIFN-α-2a therapy. No severe, life-threatening opportunistic infections were observed in any of our five patients during the course of treatment with rIFN-α-2a. The two patients who died during therapy were victims of the tumour itself that caused cachexia. No extracutaneous manifestation of KS was detected but a post-mortem was refused by the families of both these cases.

Long-term continuous rIFN-α-2a therapy did not induce any serious changes in laboratory parameters. The T-helper/T-suppressor ratio and absolute T-helper counts continuously decreased in all patients and therapy with rIFN-α-2a was not able to inhibit or reverse this tendency. The therapy was generally well tolerated and the patients were able to participate in normal daily life and pursue their professions. The changes in laboratory parameters and subjective side-effects under long-term rIFN-α-2a therapy were similar to previous observations.[16,17] In our experience, it was not necessary to interrupt or stop the therapy protocol as a direct consequence of these side-effects.

We conclude that long-term treatment of HIV-associated Kaposi's sarcoma with rIFN-α-2a can induce complete tumour remission in individual cases, increase the survival time and comfort of the patient, inhibit or reduce the occurrence of opportunistic infections, and is usually well tolerated (severe haematological side-effects should be rare). In our opinion, in the treatment of HIV-associated KS, the drug must be given as early and as long as possible (probably for the rest of the patient's life), either in monotherapy or in combination with other antiviral drugs such as zidovudine, as we have proposed in another recent publication.[18]

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Induction of lesions of dermatitis herpetiformis by autologous serum

N.H.COX AND P.S.FRIEDMANN

Department of Dermatology, Royal Victoria Infirmary, Newcastle upon Tyne NE1 4LP, U.K.

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Summary In the present study various factors which contribute to the initiation of lesions in dermatitis herpetiformis (DH) were examined. Thirty-one patients with DH, seven with bullous pemphigoid, two with linear IgA disease and two healthy subjects were studied either before starting treatment or after stopping dapsone for up to 5 days. Intradermal inoculation of freshly prepared autologous serum was followed after 18–24 h by the formation of DH-like lesions in 24/31 DH patients. The lesions were erythematous papules, often with vesicles and microscopically showed papillary tip microabscesses. Serum-induced formation of lesions only occurred in patients with active DH with some spontaneous lesion formation; it did not occur in any of the non-DH controls. The formation of lesions was dose-related, declining proportionately with dilution of the serum down to 1/16. Plasma prepared by various methods of anticoagulation (heparin, citrate, EDTA) caused lesser reactions, while addition of heparin or ε-amino caproic acid (EACA), but not citrate, to serum substantially inhibited the formation of lesions. This suggested the responsible factor might be a protease. Other vasoactive agents including histamine (1–4 μg) and compound 48/80 (1–5 μg) caused normal immediate wealing. DH-like lesions occurred in only one of 13 subjects challenged with histamine and two of nine challenged with 48/80. In all these, autologous serum elicited large vesicular responses. There is a factor(s) in serum in DH which can initiate the formation of lesions. This factor appears to be activated by clotting and can be inhibited by heparin and EACA, suggesting it may be a protease.

Despite recent advances in our understanding of the pathogenesis of dermatitis herpetiformis (DH), the sequence of events leading to the formation of lesions remains unknown.[1-3] Deposition of IgA and components of the complement pathways in papillary tips[4] occurs both in clinically normal skin and lesions;[5] localizing factors must therefore be required for the initiation of lesion formation. The cellular infiltrate of lesional skin which includes both mononuclear cells[6] and eosinophil and neutrophil polymorphonuclear leucocytes,[7,8] is probably important in the pathogenesis of blister formation. These cells release a variety of proteases and toxic proteins, some of which have been identified in the blister fluid of DH.[9,10]

During a pilot study of the effect of inoculation of autologous leucocytes into the skin of patients with DH, we noted that autologous serum used as a control caused development of urticated papules or plaques about 20 h post-injection. These were clinically indistinguishable from the spontaneous DH lesions, suggesting that a serum factor was important in triggering lesion formation. In the present study we have examined the specificity of this phenomenon and commenced characterization of the serum factor.

Methods

Patients

Thirty-one patients with DH were studied either before starting treatment or after stopping dapsone for up to 5 days to allow some spontaneous disease activity. Control subjects included two with linear IgA disease, seven with bullous pemphigoid (studied before starting prednisone) and two healthy subjects. All diagnoses were based on clinical features supported by typical findings on histological and immunofluorescence examination of a skin biopsy. Local Ethical Committee approval was obtained and subjects gave informed consent.

Correspondence: Professor P.S.Friedmann, Royal Liverpool Hospital, Prescot Street, P.O. Box 147, Liverpool L69 3BX, U K

Preparation of blood fractions

All fractions were prepared from venous blood taken 2 h before inoculation. Serum was prepared by centrifugation at 1800 *g* after allowing the blood to clot in glass and to retract at 4°C for 1 h. Plasma was prepared by prevention of clotting with either sodium heparin (50 U/ml), sodium citrate (3 ml of 0·8% solution in 10 ml of blood), or ethylene diamine tetra-acetic acid (EDTA standard Sequestrene tube); after centrifugation at 1800 *g* the upper two-thirds of the plasma layer was taken to minimize contamination by platelets or leucocytes.

Inoculation procedure

Volumes (100 μl) of the blood fractions were inoculated intradermally with a syringe (0·5 ml with 10 μl calibrations) and a 23-gauge needle. Inoculations were generally at sites of predilection for spontaneous lesions in each individual, usually the extensor forearm or lower back; in patients with pemphigoid and linear IgA disease inoculations were adjacent to spontaneous lesions. Challenge sites and pre-existing spontaneous lesions were marked with an indelible skin marker pen.

The following fractions were inoculated: (i) plain serum—positive control in all subjects; (ii) phosphate-buffered saline (PBS)—negative control in all subjects; (iii) serum diluted with PBS—1/2 to 1/16; (iv) serum with addition of sodium heparin (50 U/ml), EACA (0·1 M) or sodium citrate (final concentration as for preparation of plasma); (v) plasma prepared by anticoagulation with sodium heparin, citrate or EDTA as described above; (vi) serum heated to 56°C for 30 min to inactivate complement. Although all 31 patients with DH were inoculated with autologous serum, they did not all receive the whole set of treated fractions.

Additional challenges

Thurfyl nicotinate 5% cream (Trafuril, CIBA Labs) was applied to a 2–3 cm-diameter area of skin for 30 min. An intradermal inoculation of Compound 48/80 (1–5 μg) to degranulate mast cells was given to nine subjects. Thirteen subjects were inoculated with histamine (1–4 μg), the weal margins being marked with a fine marker at 10 min and the areas measured by planimetry.

Assessment of responses

All challenged sites were scored 18–24 h after inoculation according to the following scale: 0, no detectable reaction; 1+, impalpable erythema; 2+, erythematous papule; 3+, erythematous papule with vesiculation.

Histological examination of biopsies

In eight patients biopsies were taken from serum-induced lesions and from control sites inoculated with PBS. The tissue was formalin fixed, stained with haematoxylin and eosin and examined microscopically.

Results

Effects of serum inoculation

Inoculation of autologous serum induced at least an erythematous papule (2+ or greater) in 24 of 31 patients with DH (Table 1, Fig. 1). Three subjects with strong responses (3+) gave a minimal (1+) reaction to PBS. All the subjects who failed to respond to serum inoculation had relatively quiescent disease with little or no spontaneous lesion formation. Dilution of the serum with PBS reduced the magnitude of response proportionately in all four subjects tested (Fig. 1). However, heating the serum to 56°C for 30 min did not affect the inflammatory response in the two patients tested. The results could be reproduced by repeating the challenge at a later date in all seven subjects tested. Histological examination of serum-induced lesions showed appearances typical of DH in all eight patients examined (Fig. 2).

No reaction to serum was observed in the control patients with bullous pemphigoid, linear IgA disease or in the healthy subjects.

Comparison of serum and plasma

Plasma formed by citration of whole blood caused lesser

Table 1. Intensity of serum-induced reactions

	Score			
	0	1+	2+	3+
DH ($n=31$)	7	3	19	2
LAD ($n=2$)	2	—	—	—
BP ($n=7$)	7	—	—	—

Scores: 0, no detectable reaction; 1+, impalpable erythema; 2+, erythematous papule; 3+, erythematous papule with vesiculation. DH, Dermatitis herpetiformis; LAD, Linear IgA disease, BP, Bullous pemphigoid

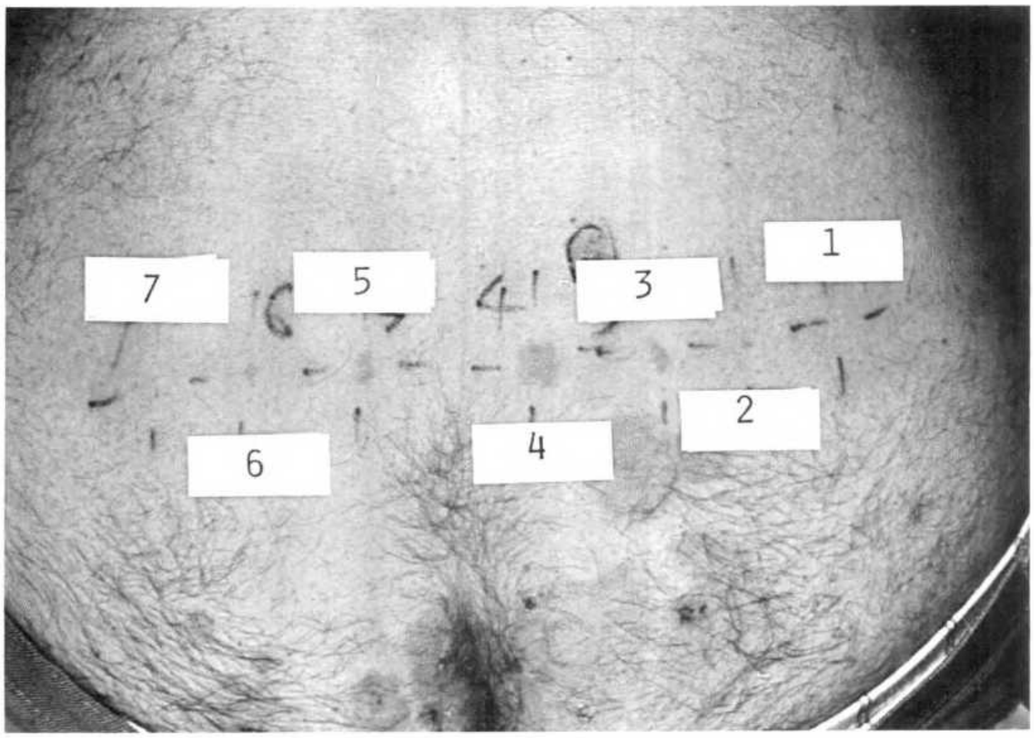

Figure 1. Effects of intradermal inoculation of serum or plasma into lower back skin of a subject with active DH. Inoculation sites are in the centre of – – marks. 1, Saline control; 2, autologous serum diluted 1/9; 3, autologous serum diluted 1/3; 4, autologous serum undiluted; 5, autologous serum plus heparin; 6, plasma prepared with citrate; 7, plasma prepared with heparin.

reactions than serum in 11/13 patients; the two who gave negative responses also failed to respond to serum. In two patients, lesions triggered by serum to which sodium citrate had been added were the same as those caused by serum with an equivalent volume of PBS added. Hence, sodium citrate inhibited lesion formation by preventing the generation or activation of the serum factor rather than via a direct action against the factor in serum.

Plasma formed by heparinization of whole blood

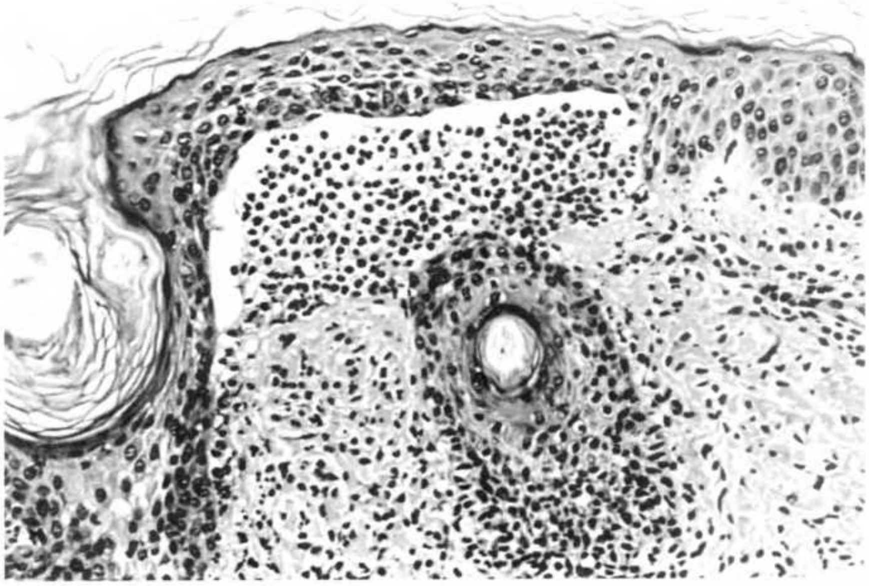

Figure 2. Lesion following inoculation of autologous serum. Papillary tip microabscess containing granulocytes and infiltrate of granulocytes (eosinophil and neutrophil) in superficial dermis. × 400.

Table 2. Comparison of responses to autologous serum, serum with added heparin and heparinized plasma

	Score			
	0	1+	2+	3+
Serum	2*	0	11	2
Serum + Heparin	6	8	1	
Heparin/plasma	6	6	3	

Fifteen DH patients were inoculated with all three fractions
*Number of reactions.

caused a lesser reaction than serum in 10/15 patients (Table 2). In four the response was completely abrogated, in three the responses were equivalent and in two the response to serum was negative. Addition of heparin to serum caused an equivalent diminution in response, indicating a direct inhibitory effect.

Plasma formed by EDTA-treatment of whole blood caused lesser reactions than serum in all four patients tested; no experiments were performed using EDTA added to serum. Addition of EACA to serum decreased the response in all five patients in whom this was performed.

Effect of thurfyl nicotinate

Application of thurfyl nicotinate cream caused mild erythema at 30–60 min in all subjects. In 14/15 patients with DH a more pronounced reaction was apparent at 18–24 h. In six of these the reaction was vesicular (3 +), in the other eight it was a red indurated plaque (2 +). Only one patient with pemphigoid had a reaction to thurfyl nicotinate at this time (1 +) and neither patient with linear IgA disease had any reaction.

Effect of wealing agents

In all patients tested the immediate weal and flare responses to histamine and compound 48/80 were equivalent to those in control subjects. Only the histamine weals were quantified: mean area 1·5 cm^2, SE 0·2 compared with 1·6 cm^2, SE 0·22 in a group of 10 healthy control subjects. However, at 18–24 h, one out of 13 subjects tested had a 2+ response to 4 μg of histamine; this subject gave a 3+ reaction to serum. Two of nine patients challenged with compound 48/80 gave delayed reactions, one of these was equivalent to the serum-induced lesion but the other was much smaller (1+ cf. 3+ for serum reaction).

Effect of body site

In all 10 patients in whom more than one site was tested, the inoculations at sites of natural predilection for DH (extensor forearm, sacral area) caused greater reactions than inoculations at other sites (flexor forearm, upper arm): median response at predilection site 2+ cf. + at other sites.

Discussion

We have demonstrated that intradermal inoculation of autologous serum in patients with DH causes development of skin lesions which are clinically and histologically identical to spontaneous DH lesions. Induction of lesions was only possible when spontaneous activity was present, such as after stopping dapsone therapy. It was not possible to induce lesions with these methods in patients with linear IgA disease, bullous pemphigoid or healthy subjects, indicating that the phenomenon is at least relatively disease-specific.

It is likely that the blisters of DH are ultimately caused by products of neutrophil and eosinophil polymorphs. The factor(s) responsible for the localization of the cellular infiltrate to produce lesions at particular sites are unknown, but cannot simply be explained on the basis of complement (C3) or IgA which are also widely deposited in clinically normal skin. In our study, there was a clear difference in magnitude of response at sites of predilection for DH compared with sites not typically affected: for instance reactions on the extensor surface of forearm were always greater than on the flexor aspect. A similar differential response has been reported for both body site and disease activity in studies of Trafuril-induced lesions in DH.[11,12]

Although Trafuril has been reported to provoke lesions in various other dermatoses including bullous pemphigoid,[11] this only occurred in one patient in our study. Trafuril causes both oedema formation (nearly analogous to plasma transudation) and a dermal polymorphonuclear cell infiltrate initially consisting of eosinophils but followed by neutrophils and leucocytoclasis.[13] It is not possible to determine which component of the complex pharmacology of Trafuril is important for induction of lesions from our experiments. It is interesting however, that Trafuril and autologous serum inoculation had similar effects in DH and a similar lack of effect in pemphigoid.

We performed a number of experiments to confirm the reproducibility of our observations and to exclude non-specific effects related to skin injury. The lack of effect of inoculation of PBS, the dose-related effects with dilution of serum and the small number of responses to wealing agents all indicate that the induction of lesions was not simply a consequence of injury.

Additional experiments have been aimed at a preliminary characterization of the serum factor capable of initiating lesions in DH. A consistent finding was that serum elicited greater reactions than plasma, irrespective of the anticoagulant used. It therefore seems that the clotting process is important for generation of the factor. The factor is not inactivated by heating at 56°C for 30 min and therefore, does not require activation of complement. This does not exclude the possibility that it is a preformed component such as C5a or C5a des arg. Addition of either heparin of EACA to serum substantially impaired the response, suggesting the relevant factor may be a protease. It is interesting that the response of DH skin to Trafuril is inhibited by pretreatment with tranexamic acid which blocks the conversion of plasminogen to plasmin;[12] and that intravenous heparin has been used as an effective therapy for DH in the past.[14]

Our observations do not indicate whether the serum factor directly causes blister formation or whether it activates secondary mechanisms from, for example, granulocytes. There is evidence for protease involvement in blister formation in various disorders including pemphigoid,[15] pemphigus[16] and epidermolysis bullosa letalis.[17] However, in most cases, the protease activity has only been demonstrated in the blister fluid, whereas the present observations suggest the presence of the proinflammatory substance in blood, which becomes activated during the clotting process. Studies aimed at the characterization of the factor are in progress.

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Cowpox/catpox infection

J.P.VESTEY, D.L.YIRRELL* AND R.D.ALDRIDGE

*Departments of Dermatology and *Bacteriology, University of Edinburgh, Edinburgh, U.K.*

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Summary A 19-year-old veterinary nurse developed a cowpox/catpox virus (CPV) infection of her right third finger following a scratch from a tom cat suffering from pneumonia. The clinical course and investigation of the infection are described and the differential diagnosis discussed.

Poxvirus infection in the domestic cat was first described in 1978.[1] Since then the incidence has increased and cats are now the most commonly reported hosts for poxvirus in the U.K.[2] Although catpox is caused by the same orthopox virus as cowpox, it is postulated that the major host for catpox/cowpox virus (CPV) is a small mammal.[2,3] CPV infection occurs mainly among 'hunting' cats in the late summer and early autumn when the putative natural hosts may be most active.[2,3] Human CPV infections have been reported sporadically since 1985[4,5] at the rate of approximately two per year but the actual incidence is probably much higher.[2] The infection is usually acquired from a sick cat, often by inoculation into a superficial wound.[6,7]

We report a case of human CPV infection acquired through a scratch from a cat which died of pneumonia. The differential diagnosis is discussed and the evidence which supports the clinical diagnosis is presented.

Case report

A 19-year-old veterinary nurse was referred to the Department of Dermatology in August 1989 with an inflamed nodule on her right third finger. Sixteen days previously she had been scratched at this site by a tom cat which she was nursing. She had developed local erythema and oedema, which spread to involve the whole finger, followed by vesiculation along the line of the scratch. Two days later she became unwell, felt feverish and consulted her general practitioner who noted tender right axillary lymphadenopathy in addition to the symptoms and signs described above. Oral erythromycin, 250 mg four times daily, for 1 week was prescribed and she was given a booster dose of tetanus toxoid. Examination of vesicular fluid by electron microscopy failed to reveal virus particles; *Staphylococcus aureus* was cultured from bacteriological swabs. The oedema and lymphadenopathy increased over the next week, despite a change of antibiotic to ciprofloxacin, 500 mg twice daily, to which the cultured organism was sensitive. She was therefore referred to the dermatology clinic.

A clinical diagnosis of an inoculation infection with a poxvirus, most probably orf, was made at the clinic and the lesion was treated with topical 2% aqueous eosin as an antiseptic and a second course of oral erythromycin for 1 week. The acute inflammation settled rapidly and a crust developed at the site of the lesion (Fig. 1). During the next week, on 1 September 1989, a serum sample was taken. The crust separated 14 days later but examination of it by electron microscopy again failed to reveal virus particles, attempts to isolate virus in tissue culture were unsuccessful and inoculation of suspensions of the pulverised crust into a sheep produced no clinical signs. A small pyogenic granuloma developed at the site of the lesion after the crust was shed but it responded to a single treatment with liquid nitrogen cryotherapy on 2 October 1989, when a second serum sample was also collected. The lesion completely healed after another month, leaving a small scar.

At the time of referral to the clinic the appearance of the lesion (Fig. 1) was reminiscent of an orf virus infection. The patient worked mainly with small mammals, especially domestic pets and denied any contact with sheep, goats or cattle. She had never been vaccinated and had never suffered from orf, milkers nodes or any other poxvirus infection. The cat which had scratched her was an 8-year-old male which lived in suburban Edinburgh and had apparently been well until 3 days before when he developed a pyrexia followed by a rapidly progressive pneumonia. The scratch occurred when he was moribund a few hours before he died. Postmortem examination revealed signs of pneumonia but

Correspondence: Dr J.P.Vestey, Department of Dermatology, Lauriston Building, Royal Infirmary of Edinburgh, Edinburgh EH3 9YW, Midlothian, Scotland, U.K.

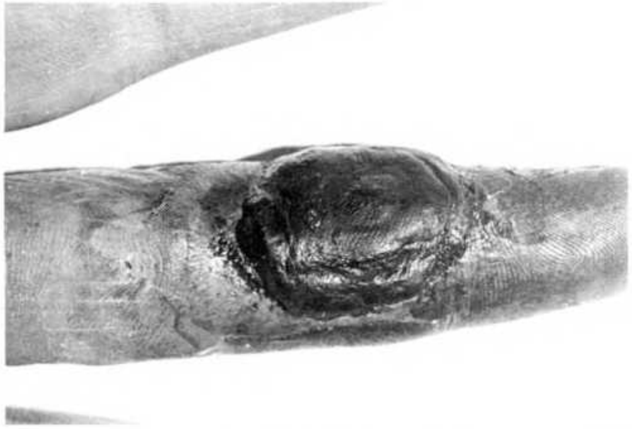

Figure 1. The appearance of the healing CPV infection when the scab had formed 23 days after the cat scratch.

bacteriological swabs taken from the lungs grew no significant bacterial pathogen. No skin lesions had been noticed on the cat but he was a 'hunter', often having caught small rodents and birds. He had also been treated for lacerations or bites on the forelimbs and muzzle on seven occasions since 1982. During the weekend before the eruption, the patient and several friends had attempted to rescue a deer wounded in a road traffic accident in Perthshire, during which her scratched hand had contacted the animal's mouth and had been contaminated with blood and saliva.

Laboratory investigations

Purified orf virus was prepared by density gradient centrifugation of crust material obtained from experimentally infected sheep.[8] Glycine extracts of foetal lamb muscle cells infected with vaccinia virus (Lister strain), CPV (Brighton strain) or a tissue-culture-adapted orf virus strain were prepared as described previously.[9] Antigens were separated electrophoretically on 8% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Western blotting was performed as described previously,[10] with test sera (detailed below) diluted to 1:100. An enzyme-linked immunosorbent assay (ELISA) was also performed using the same glycine extracts[9] and an anti-human IgM–alkaline phosphatase conjugate as a second antibody. The control antigen was an extract of uninfected foetal lamb muscle cells and the end point calculated as the highest dilution which gave an absorbance equal to or greater than the mean of eight wells (four control antigen, four test antigen) of a 1:25 dilution of a control serum plus three standard deviations. In order to reduce non-specific binding when glycine extracts were used as antigen in either the ELISA or Western blotting, all sera were pre-adsorbed with a lysate of uninfected foetal lamb muscle cells.

A western blot of electrophoretically separated purified orf virus probed with the patient's and control sera is shown in Figure 2. Track 1 clearly shows that convalescent serum from a patient with orf reacted strongly with

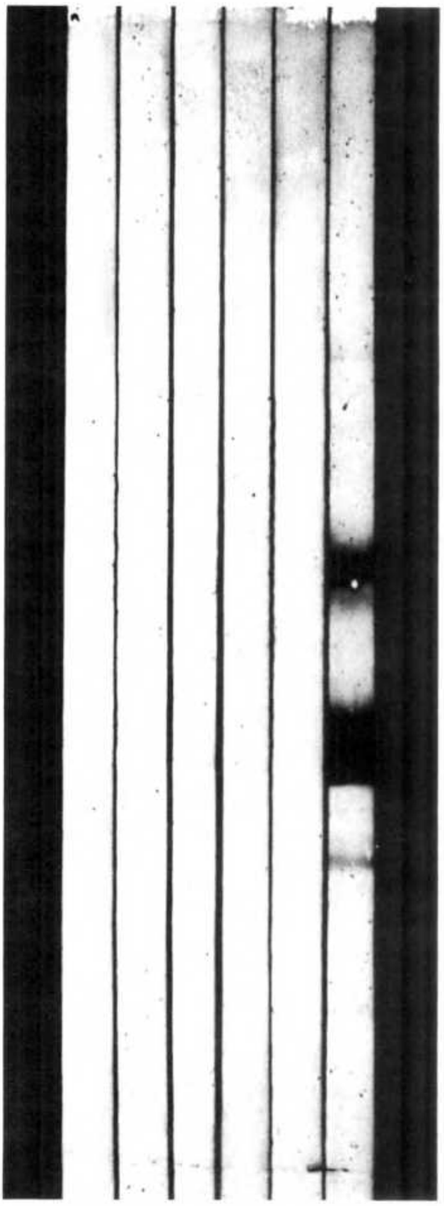

Figure 2. Western blot of electrophoretically separated, purified orf virus probed with: (1) convalescent serum from an orf infected subject; (2) index patient's serum, 1 September 1989; (3) index patient's serum, 2 October 1989; (4) poxvirus-naive human serum; (5) serum from a vaccinia vaccinee; (6) no serum.

several orf virus proteins. In contrast neither of the patients sera (tracks 2 and 3) showed any reaction. Similarly there was no reaction with serum from a patient recently vaccinated with vaccinia (track 5) nor from the negative control sera (tracks 4 and 6).

The ELISA confirmed that the patient had recently suffered an orthopox infection. The antipox IgM titre of 1/400 detected in serum collected on 1 September 1989 declined to < 1/25 by 2 October 1989. The titre was the same whether vaccinia or cowpox was used as the test antigen.

In order to define the serological reaction further the Western blotting technique was reapplied. A complex picture was found of cross-reactivity between sera and glycine extracts of virus-infected foetal lamb muscle cells (Fig. 3). Control serum from a poxvirus-naive subject (A) showed no reaction with any extract. Serum from a patient with orf (D) reacted with a single determinant from the orf virus extract (track 4). All test sera (B, C and D) reacted with the vaccinia extract (track 2). Interestingly, serum from a patient who had suffered from orf but had never vaccinated (D) showed reactivity to

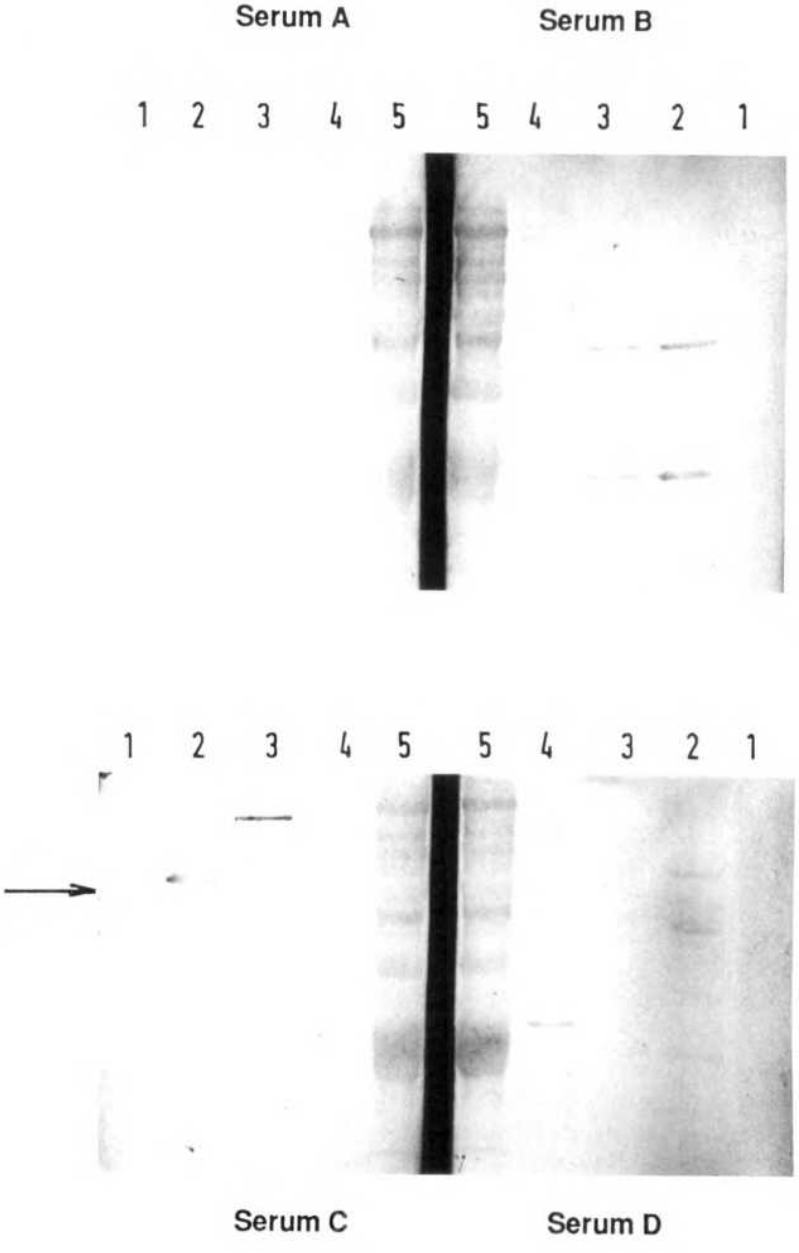

Figure 3. Western blot of electrophoretically separated glycine extracts of foetal lamb muscle cells infected with: (1) uninfected cells, (2) vaccinia virus, (3) CPV, (4) orf virus; probed with serum from: (A) poxvirus-naive control subject, (B) index patient, (C) subject vaccinated with vaccinia, (D) patient with orf infection. Track 5 contains the standard molecular weight markers.

vaccinia virus extract (track 2). Clearly vaccinia and cowpox share three major antigenic components, detected by the vaccinia positive serum (C, compare tracks 2 and 3); this serum reacted even more strongly with another band unique to vaccinia (arrowed). It is the lack of detectable reactivity to this band by the test patient's serum (B, track 2) which differentiates the antibodies present from those generated in response to vaccinia virus infection.

Discussion

The finger lesion clinically resembled an orf virus infection but the absence of exposure to sheep, cattle or goats implicated the deer as a possible source of the infection. Deer are not susceptible to orf, but a closely related parapox virus infection has recently been described in deer in New Zealand.[11] This has not been reported in Europe however, and the total lack of reactivity of the patient's serum with purified orf virus made this an unlikely possibility.

Attention therefore focused on the cat scratch as the probable origin of the presumed pox virus infection. This presumption was based on the clinical background of the case. Firstly the lesion occurred 2 days after, and at the same site as, the cat scratch; secondly the cat was a known 'hunter' and had suffered many previous bites or scratches which could have been inflicted by other cats or by his prey, a feature recorded in many reports of feline CPV infection; and finally the infection occurred during the season of peak prevalence of CPV infection.[2,3]

Attempts to isolate virus from the skin lesions and to demonstrate it by electron microscopy were unsuccessful. An ELISA for specific IgM indicated a recent infection with an orthopox virus but was unable to distinguish between vaccinia and CPV. Western blotting not only confirmed the presence of antiorthopox antibodies in the patient's serum, but also showed a differential staining pattern between that serum and serum from a vaccinia vaccinee, thus heavily implicating CPV as the cause for the infection. Neither sera from the patient prior to the infection nor from the cat were available so further analysis was not possible.

The true incidence of human CPV infection is unknown since not only has it received little attention in the medical press, but also the clinical features are reminiscent of orf or milker's nodes and thus most cases would not be investigated. However, the incidence of human CPV infection will probably rise if the infection rate among domestic cats continues to increase. Previously reported cases of human CPV infection have mostly occurred following contact with an animal clearly infected with CPV,[1-7] but in others the cat implicated appeared well.[12] However, skin lesions occurring on a haired animal such as a cat may not always be apparent and severe cases of feline CPV infection are sometimes complicated by pneumonia, as may have been the case here.[13]

The efficacy of vaccination against smallpox using the related orthopox virus vaccinia illustrates the degree of protection that cross-reactivity between different members of the poxvirus family affords. In this study we have also shown extensive serological cross-reactivity between CPV and vaccinia (Fig. 3). It is possible that human CPV infection will not only become more common as the incidence rises among domestic cats but that more severe infections will occur as the proportion of the population who have never been vaccinated against smallpox increases. CPV infection may also become an increasingly important zoonosis as the number of immunosupressed subjects in the population rises with the increasing prevalence of AIDS and the rising number of iatrogenically immunosuppressed patients. CPV infection may also become a threat to patients with dermatoses such as atopic eczema or Darier's disease in whom it may precipitate Kaposi's varicelliform eruption.[14]

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Epidermodysplasia verruciformis-associated papillomavirus infection complicating human immunodeficiency virus disease

T.G.BERGER, W.S.SAWCHUK,† C.LEONARDI,‡ ANDRIA LANGENBERG, J.TAPPERO, AND P.E.LEBOIT

Departments of Dermatology and Pathology, School of Medicine, University of California, San Francisco, CA, U.S A
†Laboratory of Cellular Oncology, National Cancer Institute, National Institutes of Health, Bethesda, MD, U.S.A
‡Department of Dermatology, University of Miami, FL, U.S.A.

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Summary Three males infected with the human immunodeficiency virus (HIV) were noted to have extensive flat warts of the face and/or body. In two there were also pityriasis versicolor-like lesions. Biopsies showed foamy, basophilic, distended cytoplasm in granular layer keratinocytes, characteristic of the human papillomavirus types seen in epidermodysplasia verruciformis. DNA hybridization techniques demonstrated the presence of HPV-type 8 in one patient and HPV 5 and 8 in another. Patients with immune suppression due to HIV infection may demonstrate the clinical features of epidermodysplasia verruciformis with the same potentially oncogenic HPV types.

Viral infections complicating human immunodeficiency virus (HIV) disease include those caused by the herpes viruses (cytomegalovirus, Epstein–Barr virus, herpes simplex and varicella-zoster viruses), pox viruses (vaccinia and molluscum contagiosum), and human papillomaviruses (HPV), including genital, common, and plantar warts.[1] Epidermodysplasia verruciformis (EDV) is a rare, sometimes familial disorder characterized by chronic infection with EDV-specific HPV types. It is manifested by numerous flat warts, red and brownish macules, pityriasis versicolor-like lesions, defects in cell-mediated immunity—perhaps permissive of the infection—and a high rate of cutaneous squamous cell carcinoma.[2] Immunodeficient states such as renal transplantation,[3] Hodgkin's disease,[4] and systemic lupus erythematosus[5] have been associated with lesions identical to those of EDV. We report three patients with EDV and immune suppression from HIV disease.

Case reports

Case 1

A 34-year-old Hispanic–American male homosexual was diagnosed as having acquired immunodeficiency syndrome (AIDS) based on oral and cutaneous Kaposi's sarcoma, *Pneumocystis carinii* pneumonia, and dementia. He had been treated with azidothymidine (AZT) and acyclovir, which was discontinued after 3·5 months because of neutropenia and anaemia. Three months after the diagnosis of AIDS he noted the appearance of numerous hyperpigmented papules on the chest and dorsal upper extremities. On physical examination, about 50 flat, slightly keratotic papules 2–5 mm in diameter were noted. He had one common wart on the hand and a plantar wart, but no genital warts. His family and personal history were negative for previous similar lesions or for skin cancer. Selected lesions were treated with either light cryotherapy or tretinoin cream 0·1% (two applications/day). After 3 weeks the tretinoin-treated lesions were largely cleared, but the frozen lesions persisted and there was depigmentation. After 2 months of tretinoin therapy his eruption appeared cleared. Six weeks later, hypopigmented macules resembling pityriasis versicolor appeared at many of the sites where flat warts had been (Fig. 1). Therapy with topical tretinoin again led to improvement.

Case 2

A 35-year-old homosexual male with generalized lymphadenopathy since 1980, seropositive for HIV since 1984, was diagnosed as having AIDS-related complex due to chronic night sweats, fatigue, and diarrhoea. His past history was significant for recurrent genital herpes,

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Correspondence: Dr P.E.LeBoit, Department of Pathology, School of Medicine, University of California, San Francisco, CA, 94143–0506 U.S.A.

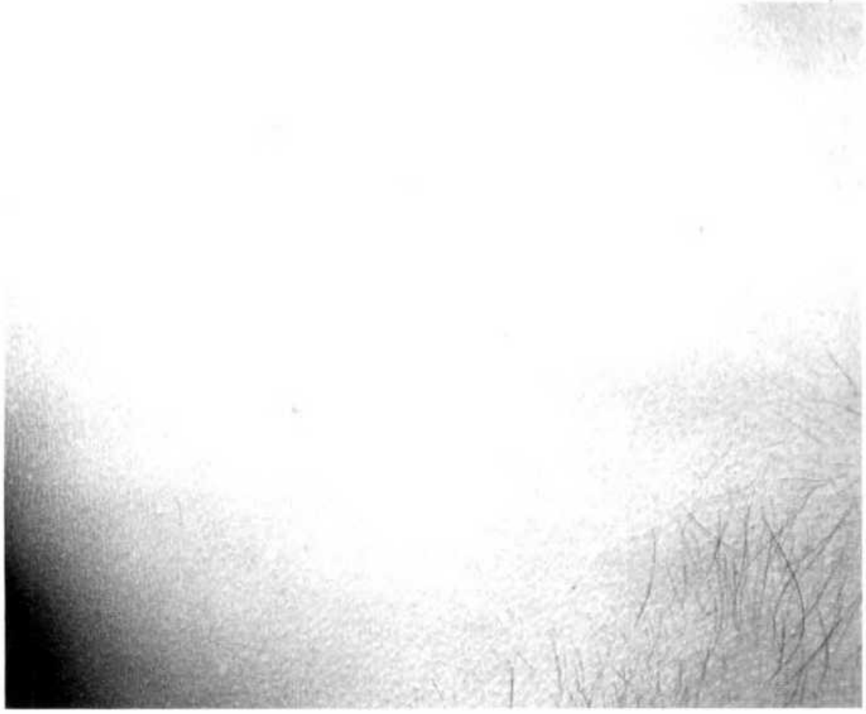

Figure 1. Case 1. Hypopigmented macular lesions resembling pityriasis versicolor on the shoulder.

rectal condylomata, and recurrent pityriasis versicolor. He first noted multiple warty papules on his hands and face in May, 1989. His family history was negative for similar lesions or cutaneous carcinomas. On physical examination, multiple 2–5 mm flat, slightly keratotic papules were noted over the dorsal aspects of both upper extremities and face. Plantar warts were present on both calcanei. The lesions were treated with tretinoin cream 0·1% for 6 weeks without benefit.

Case 3

A 28-year-old Haitian male, the sexual partner of an HIV-seropositive prostitute, developed hundreds of skin-coloured papules and hypopigmented macules on the dorsa of the hands and the distal forearms over a period of 3 months. He had oral candidiasis and a positive serologic test for syphilis. An ELISA test for antibody to HIV was positive, confirmed by Western blot. His family history was negative for skin disease or skin cancer. He was lost to follow-up.

Methods

One shave biopsy specimen from Case 1 and two biopsies from Case 2 were transported on dry ice, placed into 2 ml of lysis buffer (10 mM/l Tris, pH 8·0, 10 mM/l sodium chloride, 10 mM/l EDTA, 0·5% sodium dodecylsulphate, and 50 μg/ml proteinase K) and digested at 37°C for 12 h. DNA was extracted from the digest[6] and nick-translated with ^{32}P-dCTP (2000–3000 Ci/mM, Amersham, Arlington Heights, IL, U.S.A.) as previously described.[7]

Aliquots (1 μg) of cloned HPV DNAs of known types were blotted onto BA 83 nitrocellulose (Schleicher and Schuell, Keene, NH, U.S.A.) using a dot-blot apparatus. The nitrocellulose filter was vacuum-dried for 2 h and prehybridized for 12 h at 65°C in prehybridization solution (Denhardt's solution (final concentration: FICOL 1%, polyvinylpyrrolidone 1%, bovine serum albumin (Pentax fraction V) 1% aqueous), standard saline solution (final concentration: 0·75 mol/l sodium chloride, 0·076 mol/l sodium citrate), 0·1% sodium dodecylsulphate, and salmon sperm DNA 100 μg/ml). The prehybridization solution was discarded and fresh solution along with $2{\cdot}0 \times 10^7$ c.p.m. (specific activity $2{\cdot}4 \times 10^6$ c.p.m./μg) of ^{32}P-labelled DNA from Patient 1 was added to the filter, incubated for 20 h at 65°C with diluted standard saline citrate (final concentration 0·015 mol/l sodium chloride, 0·0015 mol/l sodium citrate, 0·1% sodium dodecylsulphate), and exposed for 12 h in a similar manner.

Results

Biopsies from all the cases showed features said to be

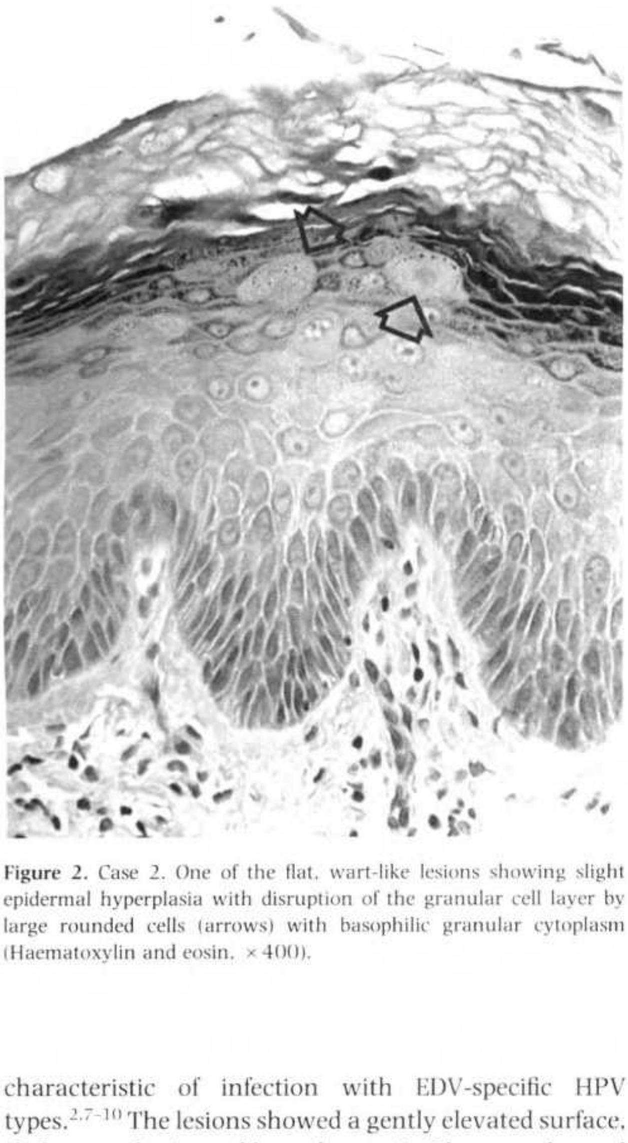

Figure 2. Case 2. One of the flat, wart-like lesions showing slight epidermal hyperplasia with disruption of the granular cell layer by large rounded cells (arrows) with basophilic granular cytoplasm (Haematoxylin and eosin, × 400).

characteristic of infection with EDV-specific HPV types.[2,7-10] The lesions showed a gently elevated surface, slight acanthosis, and hyperkeratosis. The granular and upper spinous layers contained keratinocytes whose cytoplasm was distended, basophilic, and finely vacuolated (Fig. 2). Perinuclear vacuolization and slight nuclear variability were also present. None of the specimens showed marked cytologic atypia.

The DNA dot-blot from Case 1 (Fig. 3a) showed hybridization to both HPV types 5 and 8 under conditions of low stringency. This was not unexpected, since both HPV types belong to HPV group D, and members of a given group will cross-hybridize from <1 to 40% under conditions of low stringency. However, under conditions of high stringency, DNA from Patient 1 hybridized only to type 8. DNA dot-blots from two lesions from Case 2 showed hybridization under conditions of low and high stringency to both HPV types 5 and 8 (Fig. 3b).

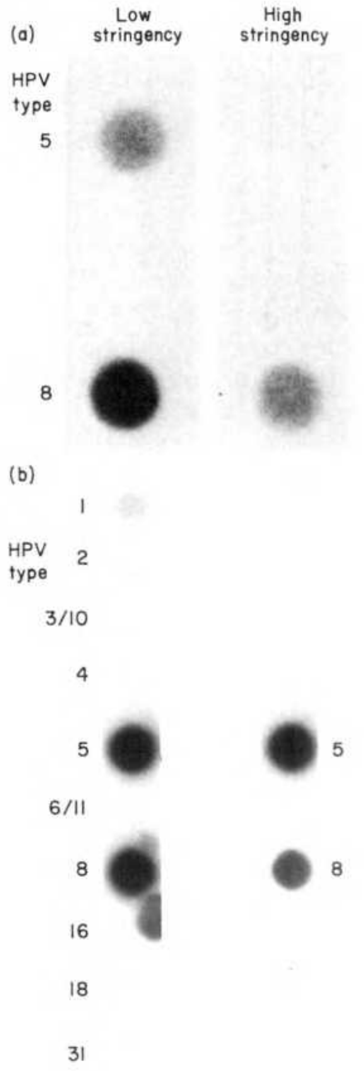

Figure 3. (a) Case 1. Dot-blot of DNA from a flat wart showing hybridization to both HPV 5 and 8 at conditions of low stringency (left), but only to HPV 8 at conditions of high stringency (right). (b) Case 2. Dot-blot of DNA from a flat wart showing hybridization to both HPV 5 and 8 under conditions of both low (left) and high stringency (right).

Discussion

This report documents that HIV-infected individuals can become infected with HPV types associated with epidermodysplasia verruciformis (EDV). The clinical lesions appeared as macules and slightly elevated papules, sometimes hypopigmented, indistinguishable from those seen in EDV. Biopsies from two of our cases had the low-power microscopical appearance of flat warts, but in the upper spinous and granular layers of the epidermis there were cells with basophilic foamy cytoplasm, characteristic for EDV.[7-10] We were able to confirm the presence of EDV-associated HPV types in the lesions of two patients by DNA hybridization, demonstrating HPV 5 and/or 8 DNA. One prior case of an HIV-infected patient whose EDV began in or before 1976 predated evidence of HIV disease by 6 years, but neither histology nor HPV typing were reported.[11] Another report documented *in situ* squamous cell carcinoma of the penis in a patient with AIDS and disseminated verrucae, but no flat warts characteristic of EDV.[12]

HPVs are a heterogeneous group shown by molecular biological techniques to recognize homology and restriction enzyme patterns. To date, more than 60 HPV types have been identified, which can be grouped according to similar clinical patterns and DNA homology.[13]

Certain HPV subtypes have been found almost exclusively in cases with EDV, a rare disorder that can be autosomal recessive although an X-linked recessive inheritance has been described in one kindred.[2,7,14] Affected individuals have extensive cutaneous HPV infection, which is often macular and hypopigmented, resembling pityriasis versicolor. Persons with EDV show abnormal cell-mediated immunity, e.g. a reduced capacity to be sensitized with dinitrochlorobenzene, decreased phytohaemagglutinin-induced lymphocyte transformation, and reduced numbers of peripheral blood lymphocytes, which form erythrocyte rosettes.[2,15] The defective cell-mediated immunity of these patients may be permissive of HPV infection. Many EDV patients develop cutaneous squamous cell carcinoma, usually in sun-exposed areas.[2,7] The DNA of certain EDV-specific HPV types (HPV 5, 8, 14) is present in these carcinomas, and in their metastases, implying that these types are oncogenic.[2,7,16,17]

Renal transplant patients have a high incidence of cutaneous HPV infection, related to therapeutic suppression of their cell-mediated immunity. The incidence increases with length of suppression, reaching 77% in patients with graft survival over 5 years.[3] These patients may be infected with all HPV types, as well as the EDV-associated types 5 and 8.[3,18] Renal transplant patients also have a high incidence of cutaneous squamous cell carcinoma on sun-exposed skin, usually appearing after 5 years of immune suppression. Sixty per cent of these carcinomas contain the DNA of HPV 5 or 8.[3] Like renal transplant patients, immunosuppressed patients with Hodgkin's disease[4] and systemic lupus erythematosus[5] may also rarely develop cutaneous HPV disease resembling EDV.

The histological and hybridization data presented here document infection of HIV-infected persons with HPV types known to be present in EDV patients. The association of these HPV types with cutaneous carcinoma in EDV and renal transplant patients makes this a finding of particular concern. More data are needed on the HPV types affecting HIV-infected persons, and whether these types are present in their mucocutaneous squamous cell carcinomas. As sunlight appears to be a significant cofactor in the development of squamous cell carcinoma in EDV and renal transplant patients,[2,3] HIV-infected individuals, especially those with disseminated cutaneous warts, should be advised to avoid sun exposure. Therapeutic ultraviolet irradiation should be used with caution in HIV-affected patients, and seems contraindicated in those with cutaneous involvement by EDV-associated papillomavirus types. As advances in treatment lengthen survival of persons with HIV infection, the expression of HPV-associated oncogenesis may become clinically relevant.

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An adult with common acute lymphoblastic leukaemia (C-ALL) presenting with skin infiltration

H.ABOUD, J.TRELEAVEN, R.CARTER,* M.O'BRIEN† AND R.POWLES†

*Departments of Haematology, *Histopathology and † Medical Oncology, Royal Marsden Hospital, Downs Road, Sutton, Surrey, U.K.*

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Summary A 22-year-old man presented with multiple raised erythematous skin lesions, pyrexia and epistaxis. A diagnosis of common acute lymphoblastic leukaemia (C-ALL) was made by morphological, cytochemical, immunological and cytogenetic examination of peripheral blood and bone marrow. Biopsy of the skin revealed leukaemic infiltration by similar cells.

Case report

A 22-year-old Iranian man presented in November 1989 with a 40-day history of general malaise and weight loss. He had subsequently developed painless, non-pruritic skin lesions, mainly affecting the face and shoulders, associated with wrist and elbow joint pain. He had experienced one epistaxis. There was no history of infection, shortness of breath or other bleeding manifestations.

On examination there were multiple raised, erythematous skin lesions of variable size on the face, shoulders and upper trunk, some of which were ulcerated (Fig. 1). Abdominal examination revealed a 3 cm palpable liver and 15 cm palpable spleen. There was bilateral axillary and groin lymphadenopathy, and diffuse subcutaneous swelling over the upper arms and back.

Investigations showed Hb 10·3 g/dl, WBC $2{\cdot}9 \times 10^9$/l, platelets 42×10^9/l; peripheral blood differential: neutrophils 77%, lymphocytes 10%, myelocytes 2% and blasts 11%. A bone marrow aspirate and trephine biopsy revealed a hypercellular marrow, virtually replaced by blast cells with no vacuolation or Auer rods. A diagnosis of acute lymphoblastic leukaemia (ALL L2) was made morphologically, and confirmed by cytochemical staining. Surface marker studies on both aspirate and trephine biopsy showed: CD 10 (J5), 90% positive; HLA-DR, 95%; CD19 (RFB 9), 97%; TdT, 90%, consistent with a diagnosis of common acute lymphoblastic leukaemia, and demonstrating the B-cell precursor nature of the blasts. Myeloid and T-lymphoid markers were negative. Cytogenetic analysis showed low hyperdiploidy with the clonal translocation t (1 : 19) more commonly associated with pre-B ALL and occasionally with some lymphomas.[1,2] A skin biopsy showed infiltration of the dermis around blood vessels and skin appendages with malignant cells (Fig. 2), the morphology and immunophenotype of which were identical to those seen in the bone

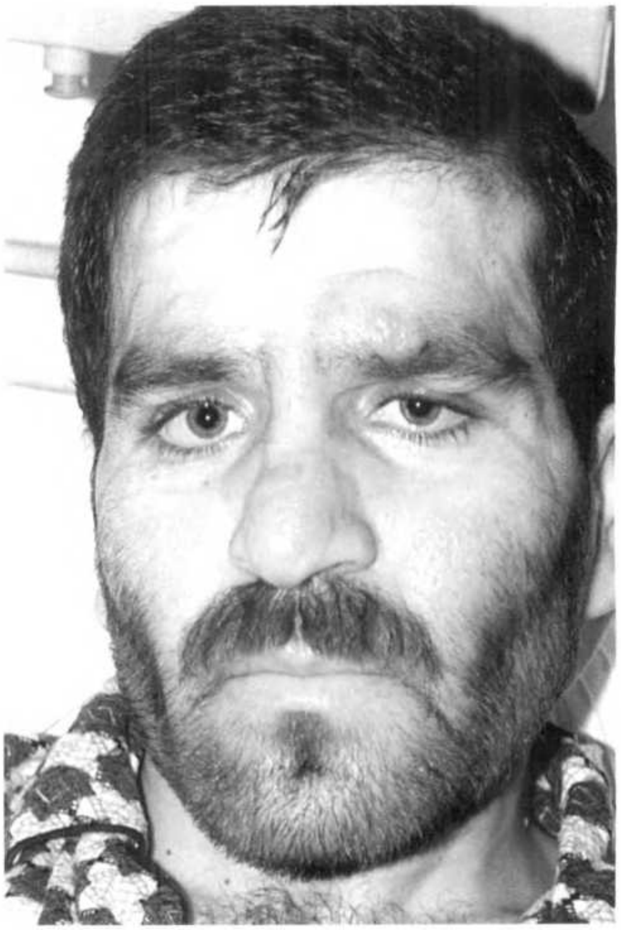

Figure 1. Lesions on the face.

Correspondence: Dr J.Treleaven.

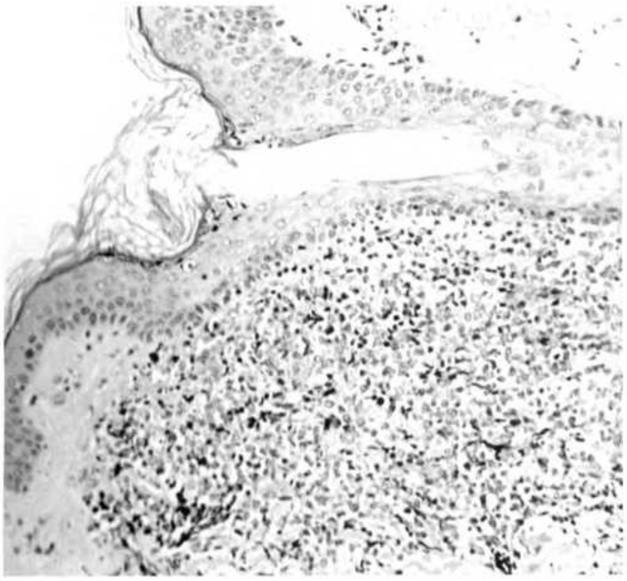

Figure 2. Infiltration of malignant cells in the dermis.

marrow. The cerebrospinal fluid was clear of disease.

The patient received UKALL-X induction chemotherapy, and early intensification. The skin lesions resolved within a few days of commencing chemotherapy. He achieved complete haematological and clinical remission by completion of induction, and is now being considered for allogeneic bone marrow transplantation.

Discussion

Infiltration of the skin is most commonly seen in association with acute myeloblastic leukaemia, particularly the monocytic and myelomonocytic subgroups.[3] It is well recognized in T-cell and B-cell chronic lymphocytic leukaemia.[4,5] We are not aware of any other case of leukaemic infiltration of the skin occurring in Philadelphia-negative, common acute lymphoblastic leukaemia presenting with skin infiltration as the predominant problem, although involvement of other extramedullary sites is well recognized.

The mechanisms regulating dermatotrophism in normal and leukaemic lymphocytes are not known. It is assumed that some T and B lymphocytes bear surface antigens which determine their tissue distribution. It is possible that the leukaemic cells from this patient exhibited undefined surface features more commonly found on T or B lymphocytes, which conferred dermatotrophism. Such behaviour would be consistent with the 'lineage promiscuity' model of leukaemogenesis described in other situations.[6] This patient exhibited 97% CD19 positivity, demonstrating that his leukaemic blasts were of pre-B nature. This feature is general among patients with CALLA-positive ALL. The chromosome abnormality present, more commonly associated with the early B-cell malignancies, supports the concept that this leukaemia co-expressed markers normally restricted to a single lineage and thereby conferred clinical features more commonly found in association with leukaemias of different lineage. There is obviously scope for defining specific cell attributes which may promote or inhibit tissue distribution of such cells.

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Unilateral naevoid telangiectasia syndrome associated with metastatic carcinoid tumour

B.E.BEACHAM AND D.KURGANSKY

Division of Dermatology, Department of Medicine, University of Maryland School of Medicine, Baltimore, Maryland 21201, U.S.A.

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Summary A patient is described who developed unilateral naevoid telangiectasia following metastases from a carcinoid tumour of the stomach.

Forty-six cases of unilateral naevoid telangiectasia syndrome (UNTS) have been reviewed and classified by Wilkin *et al.*[1,2] There are two major categories, congenital and acquired, the latter being divided into those cases that are physiological and those related to alcohol and hepatic disease. The condition is usually asymptomatic, and is seen in those disorders where there are elevated levels of circulating oestrogens. Substantial elevation of both oestrogen and progesterone receptors have been found in the lesional skin of a pregnant woman with UNTS.[3] The condition has been observed in patients with chronic liver disease and alcohol-related cirrhosis of the liver.[4,5] We describe a patient who developed symptomatic UNTS following liver metastases from a carcinoid tumour of the stomach. Measurements for oestrogen and progesterone receptor activity were performed on involved and uninvolved areas of skin.

Case report

A 69-year-old diabetic Caucasian female was seen in September 1989 with a 2·5-month history of intermittent, progressive, erythematous warm flushing eruptions on the right arm, shoulder, upper back and on the lateral chest wall. Two years previously the patient had been investigated for abdominal pain and diarrhoea which was considered to be secondary to diabetic neuropathy. No rash was noted on physical examination and she was treated at that time with diphenoxylate hydrochloride with atropine sulphate and psyllium hydrophilic mucilloid. She had no past history of congenital vascular malformations and her diabetes was controlled with glyburide. She had a previous history of mild migraine headaches, hypertension, diverticular disease and iron-deficiency anaemia.

On examination, there was striking telangiectasia involving the skin on the right side of the upper back, shoulder, upper chest and right arm involving dermatomes C6–C8 (Fig. 1). These telangiectases blanched under diascopy and filled from central puncta. Darier's sign was negative and no purpura or jaundice was noted. There was no telangiectasia of the mucous membranes and the heart and lungs appeared to be normal. There was no hepatosplenomegaly.

Laboratory investigations showed a white blood cell count of $3·6 \times 10^9$/l, a haematocrit of 31·6, serum iron of 17·0 (normal 35–140 μg/dl) and TIBC of 246 (normal 245–400 μg/dl). Serum vitamin B_{12} was 110 pg/ml, serum folate was 20 μg/ml (normal 1·9–9·8 μg/ml). Liver function tests were within the normal range except for an alkaline phosphatase of 209 U/l (normal range 30–110 U/l).

Upper gastrointestinal X-rays revealed widespread polypoid lesions throughout the stomach and a barium enema showed mild diverticulosis of the sigmoid and descending colon. Biopsies of the skin from the right upper back and right arm showed dilated dermal capillaries without any endothelial proliferation (Fig. 2). Giemsa stain was negative for mast cells and immuno-

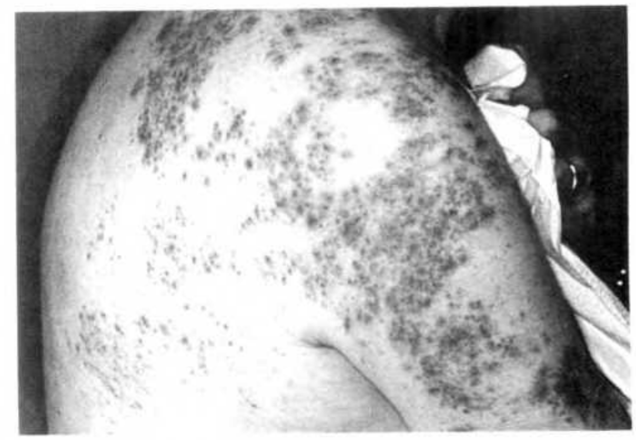

Figure 1. Erythematous telangiectatic patch on the right shoulder.

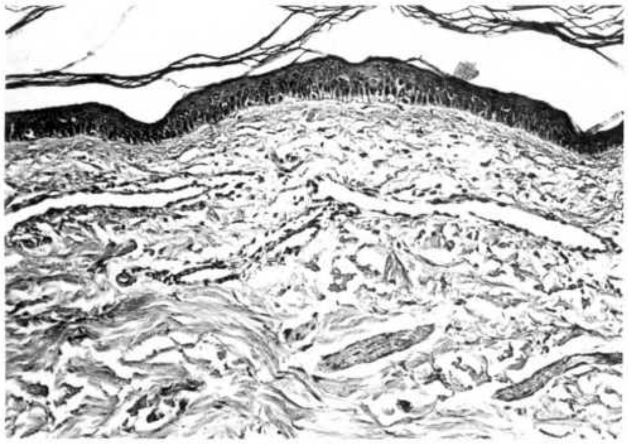

Figure 2. Dilated upper dermal capillaries with a moderate lymphohistiocytic perivascular infiltrate (haematoxylin and eosin).

fluorescence studies of the skin biopsy from the right upper back were all negative. Immunoperoxidase staining of the paraffin-embedded tissue was negative for oestrogen receptors.

A fibreoptic oesophagogastroduodonoscopy was performed and this revealed multiple large polyps over the entire gastric mucosa, but no telangiectasia was noted. Biopsy of the gastric polyps revealed infiltration with carcinoid tumour with positive silver staining. Nuclear magnetic resonance studies of the liver indicated numerous nodular areas consistent with metastases. The urinary level of 5-hydroxyindolacetic acid was 10·5 mg/24 h (normal levels $< 5{\cdot}7$ mg/24 h). The patient was commenced on intravenous 5-fluorouracil and streptozotocin as chemotherapy for her metastatic carcinoid tumour. Three weeks after the first course of treatment had been completed, the eruption had faded considerably but was still apparent. A repeat nuclear magnetic resonance scan performed 3 months after chemotherapy showed a marked decrease of the filling defects in the liver.

Biopsies were taken from involved and uninvolved skin and the tissue was snap frozen at -70°C and sent to Nichols Institute, San Juan Capistrano, CA, U.S.A. for oestrogen and progesterone receptor analysis. The involved skin was negative for oestrogen receptors (< 3 fmol/mg protein) but was positive for progesterone receptors at a level of 53 fmol/mg protein (positive results > 5 fmol/mg protein). Uninvolved tissue obtained from a symmetrical biopsy on the opposite side of the back was negative for both oestrogen and progesterone receptors.

Discussion

Unilateral naevoid telangiectasia is most commonly associated with hyperoestrogenaemic states. It is most often found in the trigeminal nerve distribution or in the C3, C4 or adjacent dermatomes.[1] Histology shows dilated vessels without endothelial proliferation in the upper and mid-dermis. The acquired variant occurs where there is an increase in physiological oestrogen i.e. during pregnancy and in women at puberty. A smaller category of the acquired variant includes those with alcohol-induced and chronic liver disease. In 80% of these cases there was no evidence of systemic involvement. There are few theories concerning the aetiology of UNTS, although it has been suggested that a humoral agent, probably oestrogen, stimulates target end organs to produce the telangiectasia. These abnormal target end organs are distributed congenitally in a dermatomal pattern.[1] Several reports have documented both cirrhosis[5,6] and chronic liver disease and portal hypertension[4] with UNTS, but no reports of this entity have been described with metastatic carcinoma in the liver.

Carcinoid tumours occur most commonly in the appendix and terminal ileum[7] although they may occur anywhere in the intestine, including the stomach.[8] They arise from argentaffin cells of the intestinal mucosa and systemic signs are seen only when liver metastases have occurred. Typical cutaneous lesions include episodic flushing of the skin, a pellagra-like dermatosis, and telangiectasia of the skin. Our patient has a previously unrecognized cutaneous manifestation of carcinoid, that of UNTS. Of interest is that she related the onset of her cutaneous eruption to the worsening of her intestinal symptoms. She complained of intermittent episodes of a warm flushing that lasted up to 30 min and occurred in the affected areas without any other symptoms. Gastrointestinal investigations at that time showed that she had widespread infiltration of her stomach with carcinoid tumour and evidence of liver metastases. A marked improvement of the UNTS was noted after her first course of chemotherapy, although the telangiectasia was still apparent.

Receptor analysis of affected tissue in UNTS has rarely been performed. Tissue from a pregnant woman showed an increase in both oestrogen and progesterone receptors in affected areas as compared to uninvolved tissue.[3] Oestrogen receptors were measured in two patients with UNTS; one specimen of involved skin showed trace oestrogen receptor activity while the other case showed no oestrogen receptors in either uninvolved or involved tissue.[7] Our analysis of oestrogen and progesterone receptors showed no evidence of oestrogen ones. Additionally, uninvolved skin from a symmetrical location on the upper back showed no evidence of either oestrogen or progesterone receptor activity. The problem with receptor analysis on UNTS stems from the relatively low percentage of the total biopsy material which represents telangiectatic vessels. It is difficult to explain the lack of oestrogen receptors if this is due to increased circulating oestrogen. It is known that normal skin contains low levels of oestrogen receptors, although variability exists based on location.[9] It is unclear whether any of the available receptor assays confer enough sensitivity to accurately evaluate oestrogen receptor levels in the skin, since their sensitivities are based on breast carcinoma tissue. It is also difficult to explain the presence of progesterone receptors in affected tissue, not only in our case, but also in the case as reported by Uhlin and McCarty.[3]

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We would like to express our gratitude to Drs Adam F.Szczypinski and Kenneth Margolis who assisted in the management of this patient.

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Erythema elevatum diutinum mimicking porphyria cutanea tarda

L.REQUENA, A.BARAT,* A.HASSON, D.ARIAS, M.C.GUTIÉRREZ, L.MARTIN AND A.DE CASTRO

*Departments of Dermatology and *Pathology, Fundación Jiménez Díaz, Clínica Nuestra Senora de la Concepción, Universidad Autónoma, Madrid, Spain*

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Summary A case of erythema elevatum diutinum (EED) closely resembling porphyria cutanea tarda (PCT) is reported. The initial skin biopsies were suggestive for PCT but porphyrin levels in the urine, stool and plasma were normal. A further biopsy from an early cutaneous lesion showed a leucocytoclastic vasculitis with fibrinoid necrosis of the vessel walls.

Erythema elevatum diutinum (EED) is a rare condition in which there are persistent plaques and nodules, particularly in relation to the arm and leg joints and in which there is a leucocytoclastic vasculitis.[1] The histology of lesions of EED is variable[2] and early lesions show a leucocytoclastic vasculitis with a dense dermal infiltrate that is largely composed of neutrophils. We describe a case of EED that clinically and histologically resembled porphyria cutanea tarda, the skin lesions being located on the sun-exposed areas of the skin.

Case report

A 35-year-old man presented with a 2-year history of bruising of the skin following trivial trauma, predominantly affecting the backs of the hands. There were erythematous papular lesions on the cheeks and forehead and numerous erosions, vesicles and bullae on the dorsal aspect of the fingers and hands (Fig. 1). Milia developed from previous bullae, mostly on the knuckles. The patient was otherwise in good health and there was no history of excessive consumption of alcohol or of previous drug therapies.

The following laboratory investigations were either normal or negative: complete blood count, erythrocyte sedimentation rate, blood chemistry studies, urinalysis, serum iron, glucose tolerance test, serum complement levels, antistreptolysin-O titre, serum immune complexes, cryoglobulins, serum immunoglobulin levels and antinuclear antibodies. Repeated estimations of urinary, faecal and plasma porphyrins were all normal.

Two skin biopsies were obtained from lesions on the dorsum of the right hand and from the right cheek. The histopathology of both specimens revealed a subepidermal split of the epidermis from the dermis and fibrosis of the papillary dermis. There was a moderate mononuclear inflammatory cell infiltrate in the reticular dermis. The tips of the dermal papillae showed eosinophilic hyaline PAS-positive deposits, predominantly within and around the walls of the capillaries. The specimen obtained from the dorsum of the right hand also showed subepidermal clefting with dermal fibrosis and milia. These findings were consistent with the clinical diagnosis of porphyria cutanea tarda.

A further cutaneous biopsy of an early papule on the forehead showed a dense inflammatory cell infiltrate that was largely composed of polymorphonuclear leucocytes in the papillary and reticular dermis. The epidermis was not involved and there was a grenz zone of uninvolved papillary dermis. Around and within the vessel walls there were fibrinoid deposits and marked leucocytoclasis (Fig. 2). Direct immunofluorescence studies of this specimen for IgG, IgM, IgA and C3 were negative.

The patient was treated with dapsone in a dosage of 100 mg daily and after 10–15 days the lesions started to resolve. After a month on dapsone the lesions disappeared except for some residual hyperpigmentation and telangiectasia on the face; no new lesions developed. After 2 months therapy with dapsone was discontinued and replaced with niacinamide 300 mg daily. The

Correspondence: Dr Luis Requena, C/ Leopoldo Alas Clarin 4-6°F, 28035 Madrid, Spain.

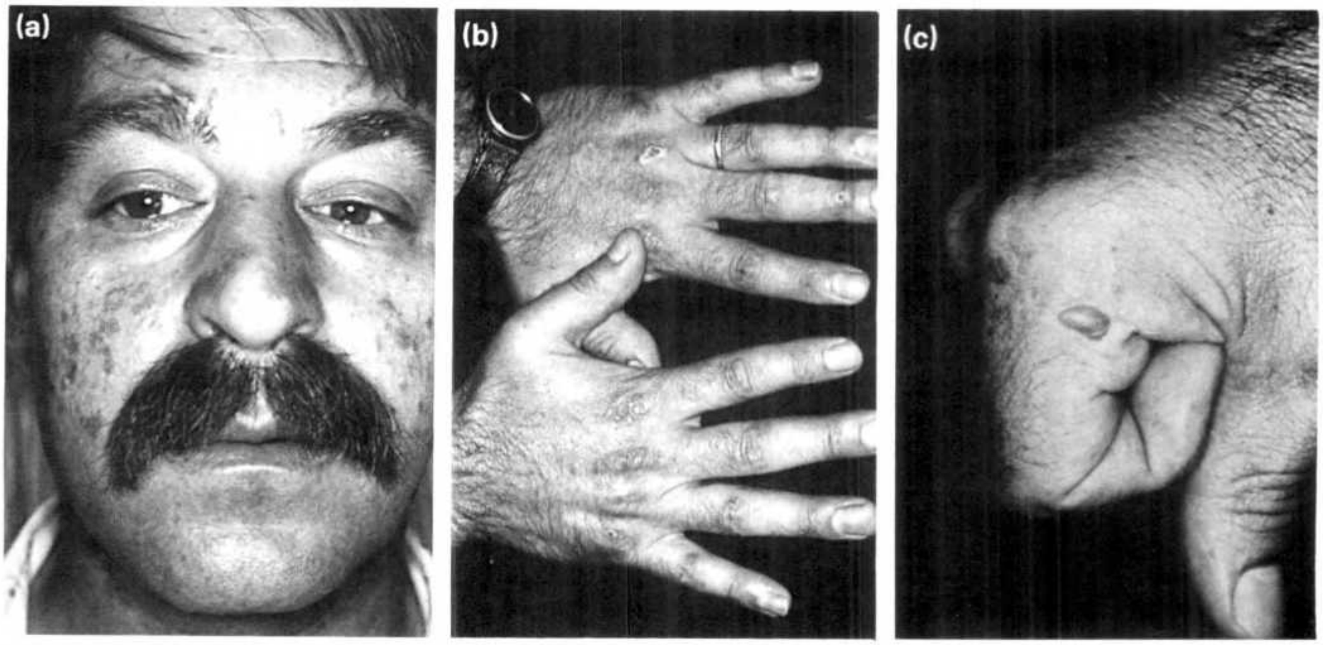

Figure 1. (a) Papular lesions on the forehead and on the cheeks. (b) Lesions on the backs of the hands. (c) Close-up of vesicular lesion on the hand.

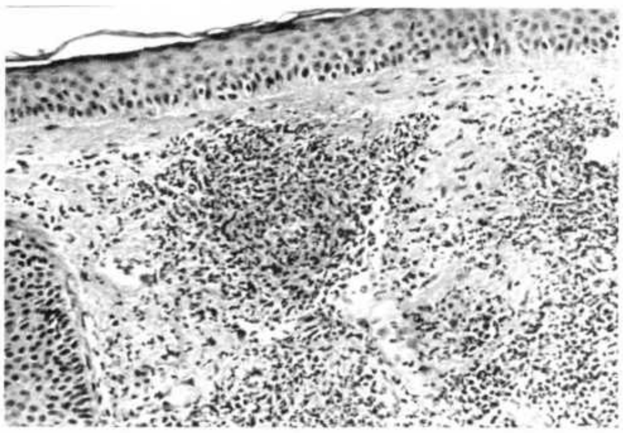

Figure 2. Histopathology of an early papule on the forehead. There are fibrinoid deposits around and within the vessel walls and abundant neutrophils with nuclear dust.

patient has remained well and no new lesions have developed.

Discussion

Erythema elevatum diutinum is usually characterized by erythematous persistent plaques and nodules that are located mainly on the extensor surfaces around the joints of the hands, arms and legs. Bullous lesions have occasionally been described in EED.[1,3–7] In our patient bullous lesions and milia were predominant, and these skin lesions suggested a diagnosis of porphyria cutanea tarda. Although the histopathology of the initial skin biopsies was consistent with the diagnosis of PCT, repeated porphyrin studies were normal. Our patient demonstrates that the histopathology of EED can be very variable according to the evolutionary stage of the lesions.[2] Clinical information concerning the stage of the lesion in EED is important if the dermatopathologist is to make the right diagnosis. Early lesions of EED usually show a leucocytoclastic vasculitis with capillary proliferation, whereas biopsies from more developed lesions show fibrosis. In our patient the biopsies of the blisters were in fact late lesions and there was no evidence of a vasculitis and neutrophilic infiltrate characteristic for EED.

The precise pathogenesis of the bullous lesions in EED is uncertain. The most likely mechanism is similar to that proposed for the vesicular lesions seen in other types of leucocytoclastic vasculitis and due to the deposition of immune complexes, particularly in the walls of small blood vessels.[8] This is associated with activation of the complement cascade and the production of C5a, a chemotactant for neutrophils. Neutrophils release lysosomal enzymes that result in damage to the walls of the blood vessels. This then results in subepidermal vesiculation.

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Benign persistent papular acantholytic and dyskeratotic eruption: a case report and review of the literature

TH.VAN JOOST, V.D.VUZEVSKI,* B.TANK AND H.E.MENKE†

*Departments of Dermato-Venereology and *Clinical Pathology, Erasmus University, Rotterdam*
†Department of Dermatology, St Franciscus Hospital, Rotterdam, The Netherlands

Accepted for publication 16 July 1990

Summary We report a case of a 35-year-old female with a persistent pruritic acantholytic and dyskeratotic eruption on the chest and vulva. The light and electron microscopic studies showed suprabasal epidermal clefting with acantholysis and dyskeratotic cells. We suggest that the most appropriate term for this case is that of benign persistent papular acantholytic and dyskeratotic eruption.

Several different papular or papulo-vesicular conditions with acantholysis and accompanied by various degrees of dyskeratosis have been described.[1–10] Except for the condition of transient acantholytic dermatosis (Grover's disease),[4] all the other dermatoses usually followed a more or less chronic course (Table 1).

There have been several reports of a papular eruption with acantholysis and dyskeratosis that is restricted to the vulvocrural areas[10,11–17] (Table 2). These cases have been described as atypical vulval Hailey–Hailey disease[11–14] or vulval warty dyskeratosis.[15] We report a patient with a persistent papular acantholytic and dyskeratotic eruption that involved the submammary areas and the vulva.

Case report

A 35-year-old female patient had a persistent history, of more than 3 years, of discrete and partly symmetrical papular lesions in the submammary region and, at the same time, more itchy and painful papules on the inner aspect of the labia majora. There was no family history of similar eruptions. The vulval lesions were clearly defined, flesh-coloured, partly grouped papules that measured 3–5 mm in diameter and some of them were eroded (Fig. 1). There were slightly keratotic discrete, light-brown skin lesions in the submammary region that clinically resembled Grover's disease. The more painful vulval lesions were excised. There were no lesions in the perianal region or in the mouth. Swabs taken from the patient and her sexual partner showed no evidence of *Candida* infections.

Histology

Light microscopy studies of biopsies from the submammary regions showed marked acantholysis and minimal

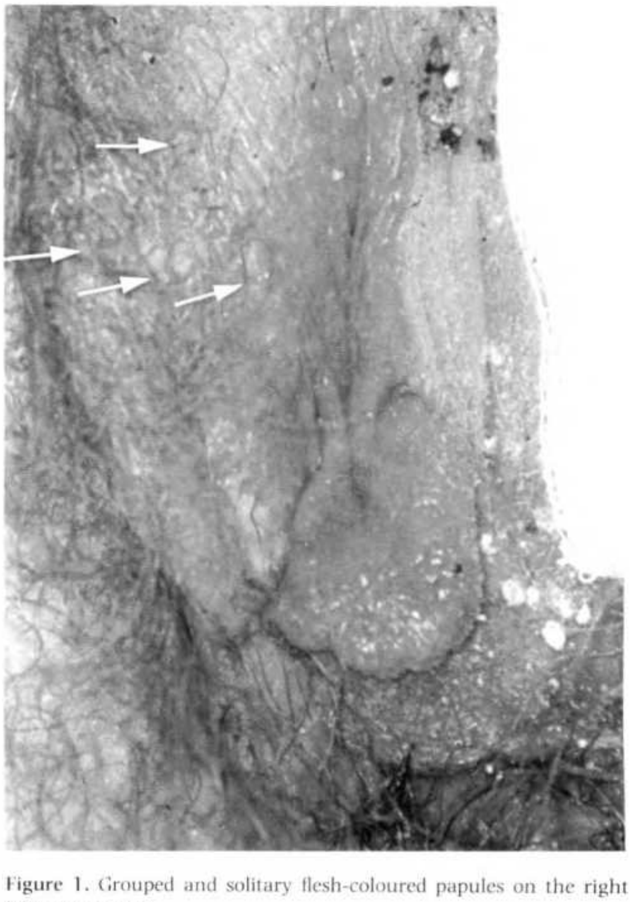

Figure 1. Grouped and solitary flesh-coloured papules on the right labium (arrows).

Table 1. Acantholytic eruptions with combined variations in dyskeratosis

Reference	Classification	Hereditary	Duration	Dyskeratosis (usual pattern)
1	Darier's disease	+	Persistent	Marked
2	Acantholytic dyskeratotic (zosteriform) naevus (Darier's disease?)	–	Persistent	Marked
3	Warty dyskeratosis	–	Persistent	Marked
4	Grover's disease (transient acantholytic dermatosis)	–	Transient	Moderate, Minimal or lacking
5	Hailey–Hailey disease	+	Persistent	Minimal
6	Acantholytic squamous cell carcinoma	–	Persistent	Minimal
7	Persistent acantholytic dermatosis*	–	Persistent	Moderate
8	Persistent acantholytic dermatosis related to actinic damage*	–	Persistent	Moderate or marked
9	Benign papular acantholytic dermatosis (BPAD)*	–	Persistent	Minimal or lacking
10	Acantholytic dermatosis* localized to the vulvocrural area	–	Persistent	Minimal or moderate

* Descriptive (provisional) diagnosis.

Table 2. Acantholytic and dyskeratotic lesions of the vulvocrural region

Reference	Number of cases	Age (years)	Provisional classification	Duration (months)	Other location
10	6	28, 37, 47, 53, 63, 83	(Distinct syndrome?) Acantholytic dermatosis of the vulvocrural area	12–36	No
11	1	22	Hailey–Hailey disease	12	No
12	1	29	Hailey–Hailey disease	7	No
13	1	39	Hailey–Hailey disease	12	No
14	1	38	Hailey–Hailey disease	?	No
15	3	49, 38, 52	Warty dyskeratosis	6–24	No
16	1	29	Papular acantholytic dyskeratosis of the vulva (Hailey–Hailey or Grover's disease or distinct syndrome?)	72	No
17	1	47	Papular acantholytic dyskeratosis	36	No
Present case	1	35	(Distinct syndrome?) Benign persistent papular acantholytic dyskeratotic eruption	36	Yes

dyskeratosis. Histology of the labia majora lesions showed a suprabasal epidermal cleft with typical acantholytic cells. Above the split, within the epidermis, there were dyskeratotic cells (Fig. 2). Small villous-like bodies were observed consisting of dermal papillae covered with a single layer of cuboidal cells with pyknotic nuclei that projected into the cleft. There were a number of inflammatory cells surrounding the cleft.

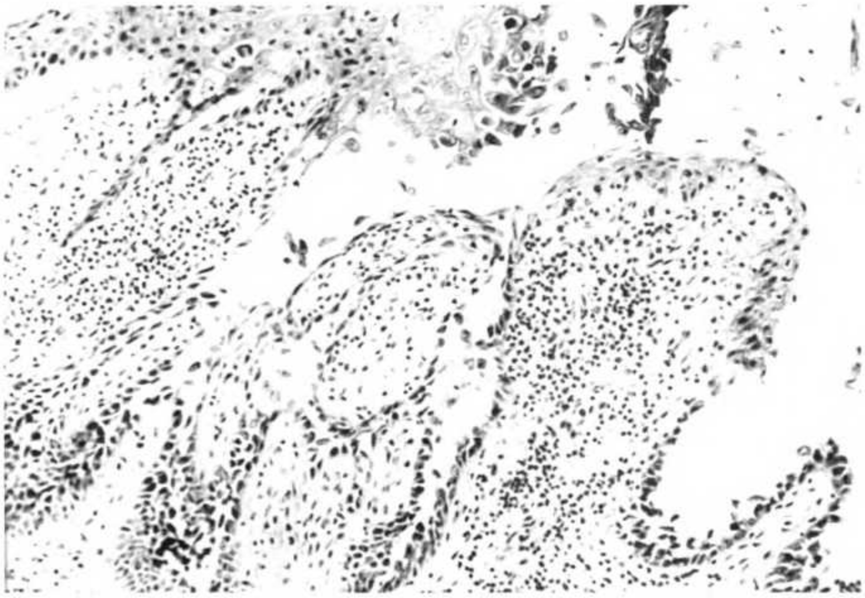

Figure 2. Histology of a papular lesion on the vulva showing suprabasal acantholysis and to a lesser extent dyskeratosis of the upper epidermis (haematoxylin and eosin, × 216).

Immunofluorescence

Direct immunofluorescence was negative with no deposition of immunoglobulins or complement in the skin.

Electron microscopy

This was performed only on the vulval lesions and the ultrastructural changes were those of acantholysis and dyskeratosis. There was a total disappearance of intercellular connections and the number of desmosomes appeared to be reduced. The tonofilaments were detached from the desmosomal plate, often aggregated around the nucleus. The tonofilament aggregations were dense, wavy and band-like and were mostly observed in the cells of the Malphigian layer, but also in isolated cells within the cleft (Fig. 3).

Cytokeratin studies

These were carried out on the vulval lesions using commercially available monoclonal antibodies and using methods described previously[18]. Staining for antipolykeratin was observed but there was no staining for anticytokeratin 7 (RRCK 105), anticytokeratin 18 and 19 (5D3). RKSE 60 (anticytokeratin 10) detects suprabasal cytokeratin filaments,[19] but was absent in the vulval lesions in our patient.

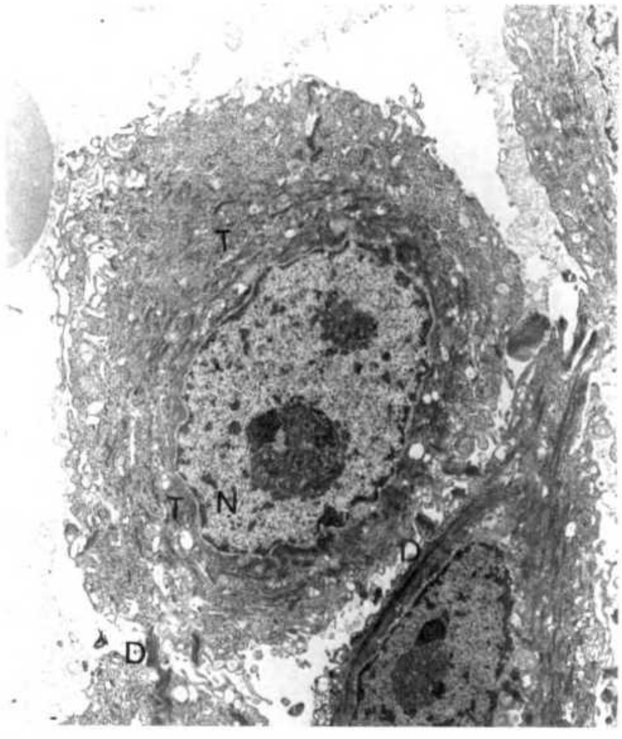

Figure 3. Electron micrograph of a vulval lesion. This shows acantholytic cells, tonofilaments (T) detached from the desmosomal plate (D) and aggregated around the nucleus (N). × 5200.

Discussion

In 1972 Ackerman[6] introduced the term 'focal acantholytic dyskeratosis' for clinical and histopathological conditions other than typical Darier's disease. A case was reported[16] of a papular acantholytic and dyskeratotic dermatosis of the vulva and it was questioned as to whether this may be a distinct entity. Coppola *et al.*[17] reported a patient with vulval and perineal papular lesions that coalesced to form plaques and this case was described as being 'papular acantholytic dyskeratosis'. Six patients were later reported with an acantholytic dermatosis localized to the vulvocrural area.[10] The histology in these cases showed acantholytic dyskeratosis that resembled Darier's disease or when there was minimal dyskeratosis, Hailey–Hailey disease (Tables 1 and 2). The presence of *Candida* in some of these cases could explain the apparent location of the vulval lesions. None of these patients had a family history and none had similar lesions elsewhere on the body as was observed in our case.

In Grover's disease and in the syndrome of benign papular acantholytic dermatosis described by Heaphy *et al.*,[9] in which lesions occurred on the trunk and the neck, dyskeratosis can be minimal or absent. The histology of the submammary lesions in our case resembled that seen in Grover's disease or that described by Heaphy *et al.*[9] The vulval lesions in our case ultrastructurally resembled the pattern described for transient Grover's disease.[20,21] The combination of the clinical features, persistent nature and absence of family history with the histopathology of the submammary lesions, however, resemble more closely that of the acantholytic and dyskeratotic type of benign papular acantholytic dermatosis. Our patient had not only vulval but also submammary lesions. A further finding of interest in our case was that staining with antipolykeratin monoclonal antibody was observed, but that cytokeratin 10 was absent.

Although this is a report of a single case, we conclude from the clinical and histopathological findings that our patient is best classified as having a benign persistent papular acantholytic and dyskeratotic eruption.

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Acquired dermal melanocytosis of the face and extremities

A.HIDANO AND K.KANEKO

Department of Dermatology, Tokyo Women's Medical College, Shinjuku, Tokyo 162, Japan

Accepted for publication 13 July 1990

Summary Four cases of dermal melanocytosis with symmetrical areas of hyperpigmentation involving the face and extremities are reported. Light and electron microscopic studies showed changes similar to those seen in naevus of Ota.

Dermal melanocytosis is seen in various congenital conditions, mostly in children, but occasionally it persists into adulthood. However, acquired dermal melanocytosis can start in adult life. Symmetrical hyperpigmentation of the skin on the face in middle-aged women is common, particularly in Asians. The clinicopathological features of this disorder have been well documented.[1,2] However, these pigmentary changes were limited to the face. We describe four cases of acquired dermal melanocytosis of the extremities that occurred with hyperpigmentation of the skin on the face.

Case reports

Case 1

A 33-year-old Korean woman had noted bilateral hyperpigmentation of the face at the age of 25. The pigmented macules had gradually increased in intensity and extent and, at the age of 31, slight pigmentation also appeared on the dorsum of the hands and forearms. There were symmetrically arranged spotted brown macules on the face (Fig. 1). A violaceous colour was noted on the alae of the nose. There was no pigmentation of the mucosa of the eye or mouth. Faint brown spots, 1–2 mm in diameter, were symmetrically grouped on the dorsa of the hands and on the extensor surface of the forearms and arms. Several faint grey–brown spots were present on the radial side of the palmar creases.

Case 2

The 31-year-old sister of Case 1 had similar pigmentation of the face since the age of 20. Soon after the development of facial hyperpigmentation she noticed slight pigmentation of the arms that gradually extended to the dorsa of both hands. Neither her younger sister and brother, nor her parents, had such pigmentary anomalies. She had a number of violet–brown macules on the cheeks and on the alae of the nose (Fig. 2). Faint brownish spots were noticed on the dorsa of the hands and on both palms (Fig. 3). There were a number of small brown spots on the extensor surface of the forearms and arms.

Case 3

A 26-year-old Japanese woman presented with hyperpigmentation of the face and extremities. It appeared on

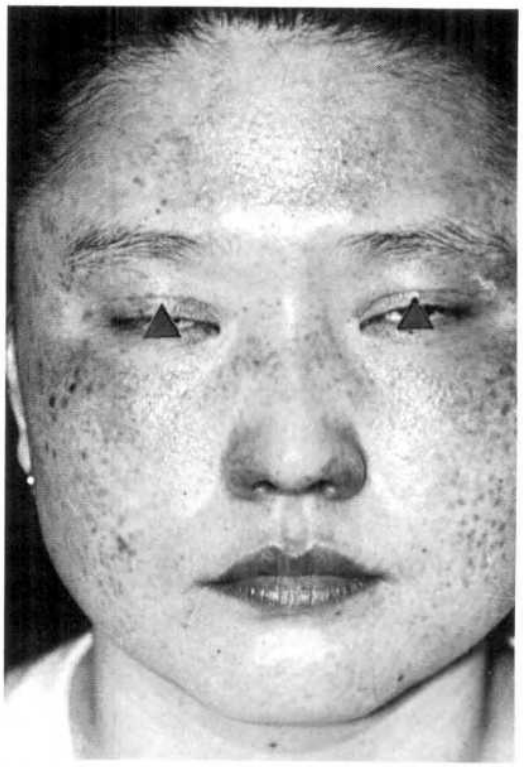

Figure 1. Brown spotted pigmentation on the face (Case 1).

Correspondence: Dr A.Hidano.

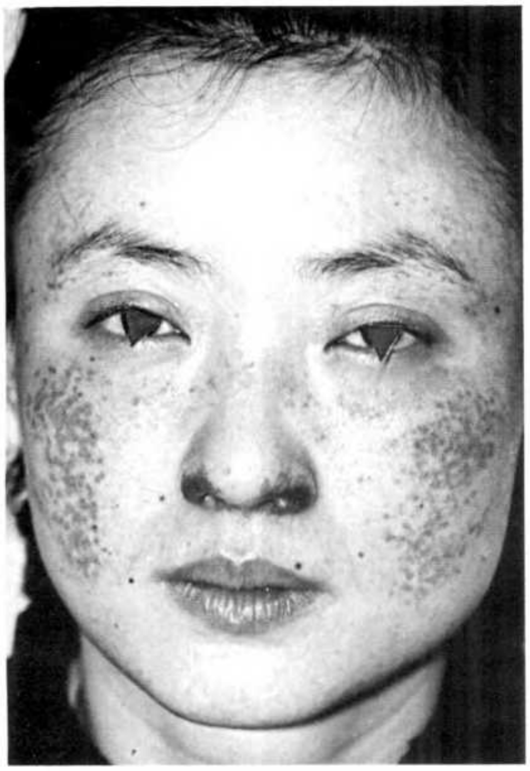

Figure 2. Violaceous brown spots of pigmentation on the face (Case 2).

the hands at the age of 18 and gradually increased in colour. The pigmentation on the face started at the age of 24 and on the palms a year later. There were a number of grey-brown spots, 1–2 mm in diameter, scattered over the root of the nose, both cheeks, eyelids and the alae of the nose. These were admixed with freckles, but were distinguished from them by their ill-defined outline. Light brown macules were irregularly scattered over the extensor surface of the arms and on the dorsa of the hands and feet. Short linear or angular brown spots were noted on the thenar and palmar creases.

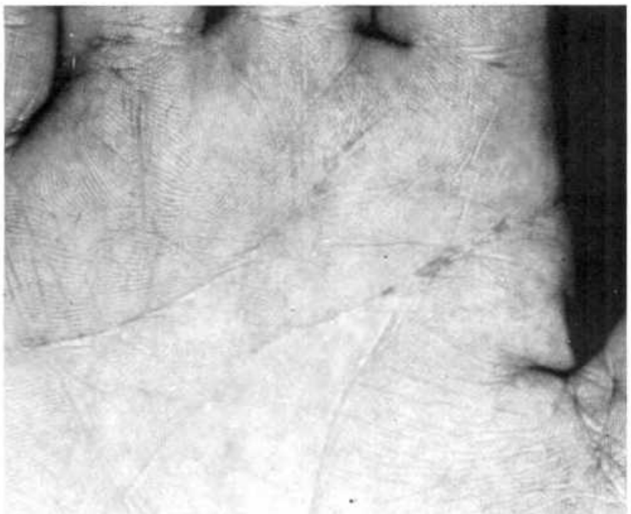

Figure 3. Faint brown spots of pigmentation in the palmar creases (Case 2).

Case 4

A 21-year-old Japanese man noticed hyperpigmentation of the nose at the age of 17 years and then, at the age of 18, hyperpigmentation of the hands. Grey–brown spots were scattered over the alae and on the tip of the nose as well as on the helix of both ears. Grey–brown macules 1–2 mm across were also symmetrically arranged on the volar side of the fingers and on the palms and plantar arches.

Skin biopsies were taken from the face of Cases 1 and 4, and from the palm in Cases 1 and 3, from the upper arm in Case 3 and from the finger and sole of Case 4. The specimens obtained from the face and palm of Case 1

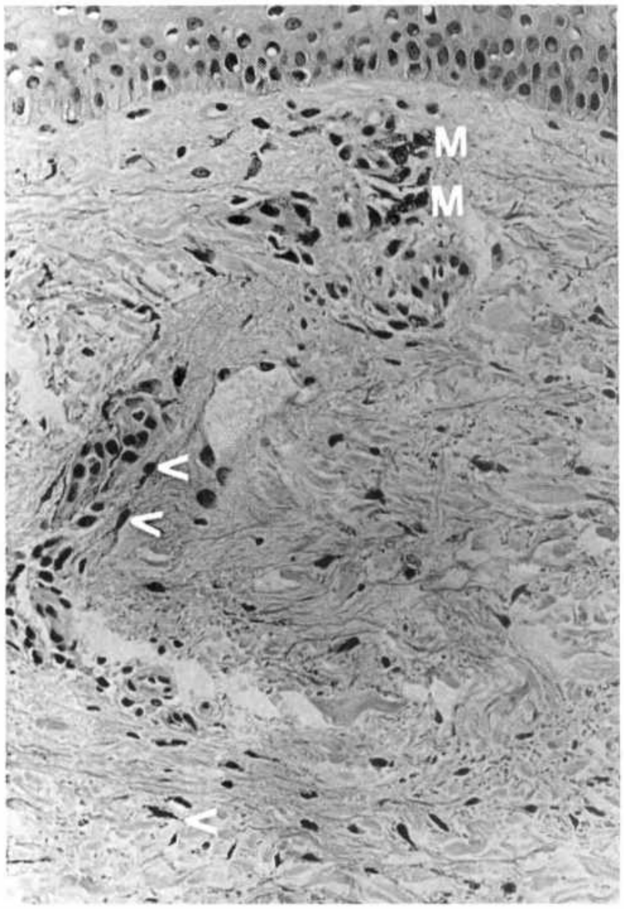

Figure 4. Dermal melanocytes (arrows) in the upper dermis and several melanophages (M), haematoxylin and eosin, × 196.

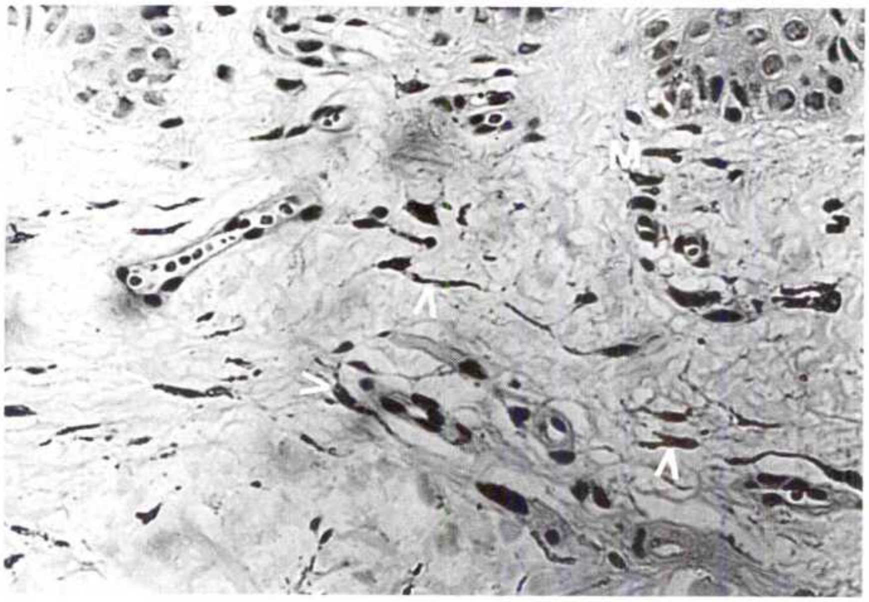

Figure 5. Elongated dermal melanocytes (arrows) and few melanophages (M), haematoxylin and eosin, × 235.

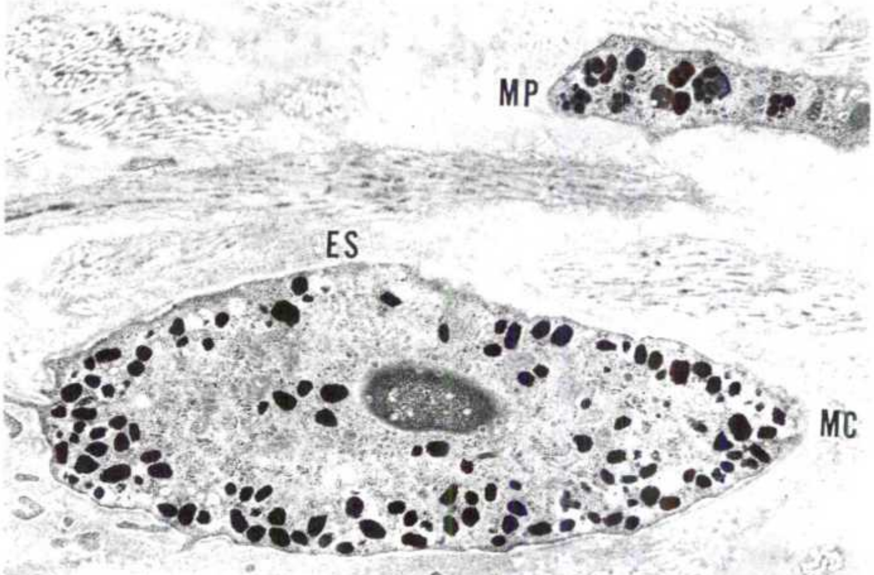

Figure 6. Electron micrograph of a dermal melanocyte (MC). The cytoplasm is surrounded by an extracellular sheath (ES) and contains many solitary melanosomes of various sizes. A portion of a melanophage (MP) is also present. × 4680.

were fixed in glutaraldehyde and processed for electron microscopy. The histopathological findings were similar in all the biopsy specimens. In the upper dermis there were elongated or fusiform dermal melanocytes filled with melanin granules. They were unevenly distributed between the collagen bundles and especially around small blood vessels (Figs 4 and 5). On electron microscopy there were spindle-shaped or bipolar cells that contained a number of single melanosomes that measured $0{\cdot}3 \times 0{\cdot}5$ μm in diameter and were mainly stage IV (Fig. 6). Melanosomes at stages II and III were rarely found. The melanocytes were surrounded by an extracellular sheath. In several cells melanosomal complexes were noted together with dispersed melanosomes.

Discussion

The acquired bilateral naevus of Ota-like macules as described by Hori *et al.*[1] are usually seen in middle-aged women and affect the forehead, temples, eyelids, zygomatic areas as well as the root and alae of the nose. In this disorder there is no melanosis of the eye or oral mucosa. On histology, pigment-bearing cells which are bipolar or spindle in shape are scattered in the upper and mid-dermis. On electron microscopy these cells contain many single dispersed melanosomes in stages II, III and IV and are surrounded by an extracellular sheath. Similar facial pigmentation has been observed in Chinese subjects with similar histopathological findings. This condition was called naevus fuscocaeruleus zygomaticus.[2]

We have studied many patients with bilateral pigmentation on the face due to dermal melanocytosis[3,4] and have distinguished two types. The first is a severe form of naevus of Ota that presents from birth with intense asymmetrical hyperpigmentation of the skin of the face accompanied by melanosis of the eye, nose and palate. The second is a symmetrically distributed discrete pigmentation of the face with a late onset. We have provisionally called the latter type the symmetrical variety of bilateral naevus of Ota. In a study of 39 cases[5] this type was separated from naevus of Ota because of its late onset and was called acquired symmetrical dermal melanocytosis of the face. The pigmentation usually starts after the age of 20 and a familial occurrence is found in 6% of the cases. The ratio of males to females is 1:9. While the site of pigmentation in males is predominantly on the lateral part of the forehead, in females it is most frequently seen in the zygomatic areas.

The pigmentation of the extremities must be differentiated from other conditions. Mongolian spots are often observed in Japanese children at birth, but occasionally appear in the first 12 months of life. Although they may occur on the dorsa of the hands and feet they are never found on the palms and soles. Blue naevi can start at any age, but the histopathology is different. Aberrant naevus fuscocaeruleus or dermal melanocyte hamartoma[6] is a dermal melanocytosis that occurs at birth and persists for life. This consists of large often mottled, grey–blue or brownish blue macules on the trunk and extremities. The pigmentation of the extremities as seen in our cases is unusual because of the late onset and the morphology of the lesions. We propose that these cases in which there is symmetrical pigmentation of the face and extremities are described as acquired dermal melanocytosis of the face and extremities.

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REPORT OF A MEETING

The Urticarias 1990

A.KOBZA BLACK, M.W.GREAVES, R.H.CHAMPION* AND R.J.PYE*
Institute of Dermatology, St Thomas's Hospital, London
**Department of Dermatology, Addenbrooke's Hospital, Cambridge*

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This report is based on a 2-day meeting held in Cambridge in January 1990. It picks out some of the recent advances since a previous meeting in 1984.[1]

Basic mechanisms

Significant developments relevant to clinical and pathophysiological aspects of the urticarias have taken place during the 5 years that have elapsed since the previous Cambridge symposium. These include a better understanding of the heterogeneity of mast cells which reinforces the differences between skin mast cells and mast cells of other tissues, the realization of the potential importance of neuropeptides in the pathogenesis of urticaria and the role of adhesion molecules such as ICAM-1 and LFA-1 in the earlier cellular events in a developing urticarial weal. The need to encourage interaction between clinical and basic scientists in this field prompted the scheduling of the present conference.

Mast cells

Dr Church (Southampton) described his technique for isolation of viable mast cells from human skin. The ultrastructural configuration of the granules (scrolls and lattices) appeared obscured because of the increased protease content of skin mast cells containing both tryptase and chymase, located in the granules. The method enabled characterization of factors regulating release of histamine and other mediators from these cells.[2] He discussed the co-release of histamine and prostaglandin D_2 from human skin mast cells and speculated about the possible role of this eicosanoid in urticaria and other inflammatory dermatoses.[3]

He pointed out that prostaglandin D_2 and leukotriene C_4 are the only eicosanoids released in detectable amounts from human mast cells, the leukotriene C_4 being released in such low quantities as to render its pathological significance uncertain in the skin. In emphasizing the possible role of the neuropeptide substance P, derived from unmyelinated sensory nerve endings, in the pathogenesis of mast cell driven disorders, he drew attention to the ability of substance P to evoke release of histamine, but not prostaglandin D_2, from human skin mast cells, although not from mast cells of other tissues, *in vitro*. This heterogeneity of human mast cells is further illustrated by the fact that cutaneous mast cells, but not those from other parts of the body, release their mediators in response to compound 48/80, morphine or codeine. The release of histamine by substance P was much more rapid than that evoked by anti-IgE (complete within 15 s, vs. 5 min for histamine) which, unlike substance P, elicits co-release of both histamine and prostaglandin D_2, implying involvement of a different stimulus-secretion coupling mechanism in the cell. The relative roles of cyclic AMP-dependent protein kinase and diacyl glycerol-protein kinase C in signal transduction in these cells remain unclear. The existence of distinct mechanisms of mast cell degranulation in the skin has implications for the pathophysiological aspects of the disease and its treatment.

Endothelial activation

The importance of interactions between immunoregulatory cytokines and adhesion molecules in inflammatory skin disorders, including urticarias, was emphasized by D.O.Haskard (London). GMP-140 is stored within endothelial cells and is expressed on the endothelial cell surface membrane after stimulation by agents such as

Correspondence: Dr A.Kobza Black, Institute of Dermatology, St Thomas's Hospital, London SE1 7EH

thrombin or histamine. ELAM-1 (endothelial leucocyte adhesion molecule-1) is not expressed by unstimulated endothelial cells but is induced on the EC surface by interleukin-1 (IL-1), tumour necrosis factor (TNF), or lipopolysaccharide, with maximal expression at 4–6 h. The presence of ELAM-1 on cutaneous endothelium can be demonstrated in inflammatory disorders of the skin, including after ultraviolet irradiation. Thus, the expression of GMP-140 and ELAM-1 on the surface of endothelium may account in part for the recruitment of neutrophils in the early and late phases, respectively, of the inflammatory response. Expression of LFA-1 on leucocytes and the upregulation of the corresponding ligand ICAM-1 on vascular endothelium in response to IL-1, TNF and interferon gamma may also contribute to the emigration of neutrophils and lymphocytes. The *in vitro* studies which Dr Haskard described have mainly been performed using cultured umbilical vein endothelium. The application of cultured cutaneous endothelial cells to this form of analysis will facilitate the investigation of factors regulating leucocyte–endothelial interactions in the skin.[4–6]

Professor E.Panconesi and Dr T.Lotti (Florence) proposed the importance of local cutaneous fibrinolytic activity in the pathogenesis of different forms of urticaria. Skin fibrinolytic activity, measured semiquantitatively, was found to be reduced in the vicinity of affected dermal blood vessels in urticarial vasculitis. These findings resembled those previously described in other cutaneous vasculitic processes. In contrast fibrinolytic activity was found to be enhanced in some non-vasculitic forms of urticaria and they suggested that the peptide degranulation products of fibrin may be themselves pro-inflammatory, or could activate release or synthesis of other mediators.[7] However, the need for more quantitative methods for evaluation of skin fibrinolytic activity was stressed.

Professor Panconesi also discussed whether antifibrinolytic drugs such as epsilon amino caproic acid could be used in the treatment of acute or normocomplementaemic urticaria, whereas urticarial vasculitis could be treated by fibrinolytic drugs such as stanozolol. Their studies also show that endothelial tissue type plasminogen activator (MW 70,000) is released following intradermal injection of the neuropeptides substance P and calcitonin gene-related peptide (CGRP). These substances are released in the skin from nerve fibres and can provoke erythema and oedema when injected into the skin.

Local pretreatment with capsaicin or compound 48/80 and systemic pretreatment with hydroxyzine reduce substance P-dependent erythema and oedema by a mechanism not totally dependent on cutaneous release of histamine. The injection of 300 pM of substance P into the skin provokes a 120% increase in cutaneous fibrinolytic activity. The injection of CGRP alone provokes a 30% increase, while intradermal injection of substance P plus CGRP provokes a 200% increase. Capsaicin pretreatment of the skin leads to exhaustion of substance P, CRGP and other neuropeptides, with exhaustion also of tissue plasminogen activator. This suggests that in future systemic treatment with capsaicin analogues might be of value in the treatment of acute normocomplementaemic urticaria, while systemic treatment with so far hypothetical substance P analogues might be useful in urticarial vasculitis and other forms of cutaneous necrotizing vasculitis.

Neuropeptides

Dr J.C.Foreman (London) summarized the evidence that neuropeptide-containing sensory nerves (C fibres) are involved in the neurogenic inflammatory (flare) response. Because of its time course of action and the spectrum of its effects, substance P is the most likely mediator of neurogenic inflammation. Injection of substance P in human skin increases vascular permeability to produce weal and also causes flare.

A controversial aspect of neurogenic inflammation is the nature of mast cell involvement. Antihistamines (H1) suppress substance P-induced flare. Substance P also releases histamine from skin mast cells. The mast cell and its histamine may be involved in the activation of the axon reflex at the point of stimulus application. Alternatively, the mast cells at the effector end of the reflex could be stimulated by substance P released from the nerve and the released histamine could contribute to the vasodilatation of flare. Resolution of the mast-cell involvement in neurogenic inflammation remains an outstanding problem.[8]

Although substance P is a likely mediator of neurogenic inflammation, other peptides co-exist with it in the C-fibres including calcitonin gene-related peptide (CGRP) and neurokinin A (NKA). CGRP produces a delayed onset, long-lasting erythema in human skin associated with a cellular infiltration. There is, as yet, little idea of what the physiological role of CGRP may be.[9]

H3 receptors

The present status of the newly described histamine H3 receptors was considered by Dr C.Archer (Bristol). Prior to 1983 two subclasses of histamine receptors had been recognized, H1 and H2. However, in that year a third subclass was defined, mainly as a consequence of research on animal brain tissue. H3 receptors are autoreceptors, i.e. they serve the function of allowing histamine to modulate its own biosynthesis in tissues, and its release from these tissues. Thus, the synthetic histamine H3 receptor antagonist, thioperamide, enhances synthesis and release of histamine from rat brain slices, whereas N-methyl histamine, an H3 receptor agonist, has an opposite and inhibitory action on these processes. Apart from the brain, H3 receptors have been shown to be expressed in lung tissue and in relation to the synaptic junctions between autonomic nerve endings and the microvasculature. High-affinity binding studies failed to demonstrate evidence of H3 receptors in rat skin, and recent unpublished work on isolated human respiratory tract mast cells suggests that H3 receptors are not expressed on this cell type. Studies using human skin *in vivo* would be of interest, using the above mentioned agonist and antagonist, and investigation of their effects on isolated human cutaneous mast cells should be a high priority.[10,11]

Serum factors in chronic urticaria

Little progress has been made in the understanding of the aetiology and pathogenesis of chronic idiopathic urticaria. It was therefore especially encouraging to hear of some novel findings in this common and disabling disorder reported by Dr C.Grattan (London). He described the presence in the serum of patients with active disease, of pro-inflammatory activity which, on intradermal injection of autologous serum, caused an inflammatory weal and flare response within 15 min of injection, lasting around 8 h.[12–14] Histologically, the mixed inflammatory cell infiltrate closely resembled a late phase reaction. Initial characterization indicates that it is heat stable with a molecular weight in excess of 10,000, and is clearly not histamine or a C5a complement component. The possibility that it may be a cytokine or an autoantibody is being explored. Studies indicate that the presence of the pro-inflammatory serum factor is related to disease activity, and the same factor was also shown to release histamine from leucocyte suspensions of normal individuals *in vitro*. Further analysis of this serum factor may yield vital information on the immunological basis of chronic urticaria and the reason for its recalcitrance.

Late phase reactions

Professor Kay with Dr Frew (London) described some immunocytochemical studies on late phase reactions in human skin.[15–17] He pointed out that all atopic subjects show a late phase reaction to a specific antigen provided enough is injected. Thus, the threshold dose for weal formation is much lower than that for development of a late phase response.

Late phase reactions characteristically show abundant lymphocytes and some neutrophils, and the use of markers of eosinophil cationic protein (ECP) suggest that the involved eosinophils are in an activated state. Using a pan-T cell marker the reaction appears to be T-cell dependent, a population of T lymphocytes being elevated at 6, 24 and 48 h. At 24 h all T cells were CD4 (helper–inducer T cells) positive. An influx of neutrophils was characteristic of the initial stages of the late phase reactions. Monocytes showed no specific change during the course of the reactions. Evidence that the CD4 positive T cells were in an activated state was suggested by the positive reactions in these cells produced by staining with anti-CD25 (IL-2 receptor) monoclonal antibody. MHC class II (HLA-DR) antigen showed a general increased expression especially on the vascular endothelium in late phase reactions. Animal models of the bronchial late phase reactions gave comparable results and these should enable adoptive transfer studies to be carried out which would define the role of each cell type in these reactions. The potential importance of the late phase response in the urticaria is considerable.

Urticarial vasculitis, delayed pressure urticaria and chronic idiopathic urticaria all show the essential clinical and histological features of late phase reactions and the role of this immunological response in these and other forms of urticaria needs to be explored.

Delayed-type hypersensitivity mechanisms

Professor Beate Czarnetzki (Berlin) discussed the possible role in urticaria of the mechanisms better known for involvement in delayed-type hypersensitivity.

Although the immediate type, IgE-mediated mast-cell release is the classical underlying mechanism of urticarial reactions, there is increasing evidence that delayed-type hypersensitivity (DTH) mechanisms are prerequisites of, or are associated with, this process.[18–21] IgE secretion is upregulated by interleukin (IL) 4 and is down regulated by interferon (IFN).

Mast cells participate actively in DTH at several levels. They have phagocytic and pinocytic properties and have been postulated to bind antigens to their highly charged granule matrix. There is preliminary evidence that mast cells can be induced to express MHC class II antigens, thus making them potentially capable of antigen presentation and processing. Mast cells have recently been shown to express mRNA and actively to secrete a whole series of cytokines, including IL-1, -3, -4, -5 and -6, interferon-γ, granulocyte macrophage colony stimulating factor (GM-CSF) and several agents with chemotactic and pyrogenic properties. This would enable them to participate in lymphocyte activation (via IL-1), mast cell (IL-3, -4) and eosinophil (IL-4) growth and differentiation, IgE-regulation (IL-4, IFN-γ), immunoglobulin secretion (IL-6), haematopoiesis (GM-CSF), the induction of fever and the recruitment of inflammatory cells. There is good evidence that classical mast-cell mediators like histamine and heparin can up- as well as down-regulate lymphocyte transformation in a dose-dependent fashion.

Evidence that DTH-related mechanisms may also be involved in clinical urticaria is sketchy. Lymphocytes and macrophages have been shown to infiltrate weals of extended duration. Macrophage migration inhibitory factor (MIF), the first cytokine described in the literature, has been demonstrated to be expressed on endothelial cells and macrophages in various types of urticarial lesions. It has been postulated that interleukins might explain fever, leucocytosis and leucocyte infiltrates in patients with delayed pressure urticaria. Recently, reagents and the technology have become available to examine DTH mediators in urticaria in greater depth, and it is to be expected that data clarifying the role of DTH in urticaria will soon become available.

Some clinical types of urticaria

Idiopathic urticaria

Professor B.M.Czarnetzki pointed out how all the various classifications of urticaria include a category called idiopathic urticaria, but what does it imply? Does it stand for the inability, perhaps even lack of diligence, of the physician to find the underlying eliciting agent or does it encompass the types of urticaria where the mechanisms are unknown? It seems clear that most cases of chronic urticaria fit into this latter category and that it is clinically appropriate to recognize it and work out the basic mechanisms as a disease or group of diseases in their own right.

Angio-oedema

Professor M.W.Greaves classified the angio-oedema group of disorders into hereditary and acquired groups. The acquired group included acquired C1 esterase deficiency, idiopathic, allergic, angio-oedema with lupus erythematosus, with hypereosinophilia and due to physical agents.

He emphasized the more recently described forms of angio-oedema. In acquired (non-hereditary) C1INH deficiencies, a clinical picture identical with that of hereditary angio-oedema occurs. They can be distinguished by a lowered level of plasma C1 that does not occur in the hereditary form. Two groups of patients are recognized. One group has a lymphoproliferative disease.[22] Here the angio-oedema is due to circulating anti-idiotypic antibodies to the monoclonal immunoglobulin. Activation of the early components of the complement pathway leads to increased consumption of C1INH. These patients may obtain prophylactic benefit from danazol, similar to the patients with hereditary angio-oedema. In the second group IgG1 autoantibodies directed against C1INH are present in the absence of underlying lymphoproliferative disease.[23] These prevent binding of C1INH to C1. Activated C1 causes degeneration of C1INH to an inactive degradation product. These patients may benefit from treatment with prednisolone. Angio-oedema due to angiotensin convertase enzyme inhibitors, such as captopril and enalapril, can occur rarely.[24] These drugs inhibit the enzyme that is also responsible for bradykinin degradation. They therefore may potentiate bradykinin activity leading to angio-oedema and urticaria. Rarely reported syndromes include lupus-like disease associated with hereditary angio-oedema[25] and angio-oedema associated with hypereosinophilia.[26] Angio-oedema can rarely occur in most of the physical urticarias in patients who have a high level of sensitivity to the stimulus. However, angio-oedema is a characteristic presentation in the rare vibratory angio-oedema.[27]

Drug reactions

Professor H.Merk (Cologne) discussed the several mechanisms by which drugs can cause or exacerbate urticaria, for example the ACE inhibitors discussed above having a direct action on the kinin system. This occurs in one case in 3000, with four fatalities recorded. Care must also be taken with beta blockers: these can enhance allergic reactions as well as make the treatment of anaphylactic reactions by the use of adrenaline much less easy to manage. There are now various techniques to try and achieve the ambition of the past few decades to be able to detect allergic reactions to drugs *in vitro*. These include immunoblotting, the lymphocyte transformation test and incubating the drug with microsomes to produce the putative sensitizing metabolites.[28] In this way IgE-mediated reactions, for example to Trasylol and cisplatin, have been confirmed *in vitro* by his team.

Contact urticaria

Dr A.Lahti (Oulu, Finland) described several new substances to be added to the long lists of those able to induce contact urticaria. These include cellulose, gelatin, buckwheat, lime, levomepromazine, petrol, kerosene, naphtha and Na-pyrrolidone carboxylic acid (natural moisturizing factor). Aspirin inhibits the non-immunological reactions to benzoic acid and methyl nicotinate, whereas terfenadine does not. Capsaicin inhibits the erythema from histamine but not the erythema or oedema from benzoic acid or methyl nicotinate. The 3- and 4-, pyridine carboxaldehyde isomers are capable of producing immediate contact reactions but not the isomer with the aldehyde in position-2. UVB, UVA and PUVA treatment inhibit reactions but 1064 nm laser irradiation enhances reactions to benzoic acid and methyl nicotinate.

Urticarial vasculitis

Professor N.A.Soter (New York) gave an update of his paper of 1984.[1] Necrotizing vasculitis of venules presenting as urticaria/angio-oedema is a type of hypersensitivity angiitis that occurs in patients with serum sickness; certain collagen-vascular disorders, such as systemic lupus erythematosus and Sjögren's syndrome; infections, such as hepatitis B virus; certain forms of physical urticaria; after potassium iodide ingestion and as an idiopathic condition. The signature skin lesions appear as weals and/or angio-oedema, at times with foci of purpura. Other dermatological lesions include bullae and livedo reticularis. Distinctive features of the weals include their long-lasting nature (3–5 days), induration and residual hyperpigmentation. Extracutaneous manifestations include general features such as fever, malaise, and organomegaly as well as specific organ involvement, notably of the synovia, kidneys, respiratory tract, gastrointestinal tract, eyes and central nervous system.

Skin biopsy specimens show fibrinoid necrosis of venules and various numbers of infiltrating inflammatory cells. On direct immunofluorescence, immunoreactants are deposited at the basement-membrane zone and around the blood vessels. An elevated erythrocyte sedimentation rate is the most consistent laboratory abnormality. Serum hypocomplementaemia may be present or the complement system may be normal. A variety of circulating immune complexes has been detected with the most frequent being a low-molecular-weight 7s C1q precipitin identified as IgG. An algorithm of therapy includes the administration of antihistamines, non-steroidal anti-inflammatory agents, colchicine, hydroxychloroquine, corticosteroids, and immunosuppressive agents. The prevalence of urticarial vasculitis among patients with chronic urticaria/angio-oedema remains undefined. The natural history is unknown but patients have been described with episodes of cutaneous lesions for up to 25 years. In one series with a follow-up for 1 year, 40% of patients experienced resolution of skin lesions. In another study with a follow-up of 12 years, resolution occurred in only one patient. Patients with idiopathic disease have subsequently developed systemic lupus erythematosus. Deaths have been reported from pulmonary disease, sepsis, and myocardial infarction.

Systemic capillary leak syndrome

Dr Pamela Ewan (Cambridge) presented a case report of the systemic capillary leak syndrome (Clarkson's Syndrome (SCLS)). This is a rare disorder characterized by episodes of capillary leak with sudden loss of large volumes of fluid from the intravascular compartment. Angio-oedema is present in only a proportion of cases.[29,30] A 41-year-old female presented with a history of four major attacks in which she became weak and faint and eventually collapsed. The blood pressure was unrecordable. She recovered after treatment with intravenous fluids. She also had minor attacks, occurring every 4 days, with nausea, bowel action, abdominal discomfort, general malaise and weakness, which improved spontaneously. Investigations between attacks were normal apart from an IgG paraprotein, 10 g/l.[31]

The cause and pathogenesis of SCLS are not known; however, of the few reported patients, the majority have a paraprotein but no evidence of any associated diseases. There is no known treatment and the disorder can be fatal. An attempt was therefore made to treat her by lowering the level of the paraprotein. On two separate occasions plasma exchange (once in combination with and followed by immunosuppression) lowered the paraprotein level from 8–12 to <5 g/l and caused complete but short-lived remission. The subsequent rise in the paraprotein level was associated with relapse. Plasma exchange with immunoabsorption, using a protein A column to deplete IgG selectively, also caused a remission, but despite continued immunosuppressive drugs the paraprotein level rose and symptoms recurred.

This demonstrates that reduction of the paraprotein results in remission and suggests that the paraprotein is involved in the pathogenesis, possibly by generation of permeability factors, but the precise mechanism remains to be established.

Hereditary angio-oedema

Dr R.Harrison (Cambridge) reviewed the recent advances in knowledge of the basic mechanisms underlying hereditary angio-oedema (HAE). This is inherited as an autosomal dominant trait and is manifested by episodic bouts of subcutaneous and mucosal swelling. The disease is consequent on low (5–30%) levels of functional C1-inhibitor. Two forms, type I and type II, have been described. In both there exists a single gene only for functional C1-inhibitor, and the lower than expected levels of functional C1-inhibitor in plasmas of affected individuals occur because of increased catabolism of the protein. Type II HAE can be differentiated from type I by the presence of a dysfunctional protein; this is catabolized more slowly than the functional protein, producing a normal antigenic level in plasma.[32] Analysis of genomic DNA from individuals with type I HAE has shown that many have deletions of exon 4 or exon 7, probably arising as a consequence of unequal crossover between all repeat sequences that flank these exons.[33,34] The structural basis for dysfunction of many of the type II HAE proteins has also been elucidated. C1 inhibitor is a member of the serine protease inhibitor (SERPIN) superfamily. Members of this family are characterized by a 'stressed' surface fold that contains the P1 ('target') residue, the major determinant of inhibitor specificity.

In addition, many of the residues flanking the P1 site are accessible to proteolytic attack. Functional C1 inhibitors (either purified or adsorbed from plasma on to an immunoaffinity column) can be cleaved, at the P1 residue, by trypsin, whereas P1 residue mutants are resistant. This property is used to define P1 residue mutant proteins; the mutant P1 residue is identified by sequence analysis.[35] Investigation of a large number of type II kindred (28) has shown that most are either P1 histidine or P1 cysteine (the classical 'albumin-linked' variant) mutants. These two residues are those expected from inherent instability of the CG dinucleotide, contained in the P1 antigen codon (CGC), and have also been seen in a related SERPIN, antithrombin III. Single cases of P1 serine and P1 leucine mutant proteins have also been observed. In addition, a limited number of dysfunctional proteins with mutations in the stressed fold that flanks the P1 residue have been described, indicating a critical role for this region in maintaining a functional protein. The impetus for much of the analysis of type II variant proteins was the hope that fuller characterization might demonstrate some heterogeneity in the degree of dysfunction against the physiological target enzymes C1r, C1s, kallikrein and factor XIIa, and hence provide further clues as to the nature of the mediator of angio-oedema. In this respect it has so far been unsuccessful, and both bradykinin and a peptin (the 'C2 kinin') derived by unregulated C1s and plasmin action on C2 have their proponents.

Physical urticarias

Some aspects of the molecular mechanisms of the heterogeneous group of physical urticarias were reviewed by Dr Anne Kobza Black (London). Although a central role has been assigned to activation of the mast cells, directly or indirectly, by physical factors, mast-cell numbers do not appear to be increased. Recently, attention has been focused on the participation of epidermal and resident dermal and infiltrating inflammatory cells in the urticarial response.

In dermographism further support for the role of the mast cell has been obtained. Application of a potent topical steroid for 6 weeks induced a reduction in dermographometer-induced wealing associated with a reduction in mast-cell numbers and histamine content of the skin.[36] In idiopathic cold urticarias, in addition to histamine, products of arachidonic acid have been found in venous blood draining challenged skin.[37] The main cyclo-oxygenase product of mast cells, PGD_2 and LTE_4, and a stable metabolite of the major lipoxygenase product, LTC_4 of mast cells, were found in low concentrations. This suggested only a minor contribution of these pro-inflammatory mediators to the urticarial response. The role of platelets in the pathophysiology of cold urticaria was suggested by the demonstration of increased levels of the platelet-activating factor (PAF)-like lipid, the specific platelet protein factor 4 in the circulation after cold challenge, though the latter finding could not be confirmed.[38] Further support for platelet participation is the demonstration by electron-microscopy of packed platelets in blood vessels in biopsies obtained from cold-induced weals.[39]

The potential of the platelet as an inflammatory cell was reviewed by Page.[40] In delayed-pressure urticaria, there is an early dermal infiltrate predominantly of neutrophils and eosinophils and later, after 5 h, of lymphocytes and eosinophils. However, no increase of the eicosanoid chemoattractants (LTB_4, and 12-hydroxyeicosatetraenoic acid) for these cells could be demonstrated in the lesions at 6 h.[41] The eosinophil specific protein, Major Basic Protein, demonstrated by immunofluorescence in the lesions through its cytotoxic and pro-inflammatory properties,[42] may be an important mediator of the delayed weal.

Dr F.Lawlor (London) reviewed the treatment of delayed-pressure urticaria. It was emphasized that there is no satisfactory drug treatment for this condition. Antihistamine therapy is ineffective although it can suppress the associated chronic idiopathic urticaria.[43] Prednisolone may improve the majority of patients, but unacceptably high doses of over 30 mg/day may be needed to maintain good control.[43] Objective evaluation of therapy with non-steroidal and anti-inflammatory drugs in double-blind controlled studies, evaluating the subjective response and the width of pressure-induced papules with a calibrated dermographometer, has been undertaken. Neither indomethacin,[43] nor colchicine, a polymorphonuclear leucocyte chemoattractant inhibitor,[44] were shown to be effective using this method. An explanation about the avoidance of pressure and suppression of the associated chronic idiopathic urticaria using antihistamine therapy was suggested.

Recent developments in solar urticaria were presented by Dr P.Norris (London). Several groups[45,46] have detected a circulating photoallergen generated on absorption of light energy by a precursor which can be either an abnormal endogenous substance or a normal skin constituent. Removal of this factor by plasmapheresis can result in prolonged remission.[47] In some cases, exposure to long-wave visible light, before or after urticaria-inducing wavelengths, can inhibit solar urticaria.[48] These inhibition spectra which may inactivate the photoallergen or its precursor, could potentially be used as treatment at home. Treatment with a non-sedating H1 receptor antagonist generally provides a greater than 10-fold increase in the minimum wealing dose.[49] Graduated exposures to UVB, UVA and particularly PUVA can achieve a considerable increase in the minimal wealing dose, acting possibly by mast-cell stabilization or through persistent occupation of IgE binding sites by photoallergen.

Clinical investigation of urticaria

In an open discussion session, Dr R.H.Champion (Cambridge) and the participants agreed that too much time and expense are spent on a routine series of investigations and that more time should be spent on the clinical history. The same theme was presented in posters by Dr A.Anstey (High Wycombe), Dr J.R.Mekkes (Amsterdam) and Professor J.Sayag (Marseilles). Clinically, it is relatively easy to recognize some 50 variants.[1,2,50,51] For each of these, various tests may then be appropriate. If the history leads to the diagnosis of idiopthic urticaria, as occurs in 70% of cases referred to British dermatology clinics, then with occasional exceptions the following routine investigations, each of which may have its uses with a different history, should *not* be considered appropriate: skin tests; total IgE; RASTs, serum complement and immune complexes; C1 esterase inhibitor; autoantibodies; serum protein electrophoresis; urea and electrolytes; liver function tests; skin biopsy. Even finding abnormal thyroid autoantibodies in the absence of any clinical evidence of hypo- or hyperthyroidism is not very helpful for clinical management.[50,52] Tests for infections, focal sepsis and intestinal parasites were considered to give such a low yield of clinically useful results that their routine use was not recommended. Skin tests for *Candida* sensitivity are simple enough but seldom very rewarding.

What could be considered appropriate was a blood count and ESR; asking the patient to keep a food diary around any attacks; and a diet avoiding food additives for 3 or 4 weeks. At times this could usefully be followed up by a series of oral provocation tests, although it is difficult to get reliably reproducible results. It was generally agreed that a very strict elimination diet, although sometimes usefully involving the patient's active participation along the lines they are expecting, was very seldom helpful.

Treatment

Professor E.Paul (Giessen, Nürnberg) discussed the drugs available for the management of urticaria. In the absence of specific reliable antagonists of the other mediators, antihistamines remain paramount but he also emphasized the importance of proper dialogue with the patients and not just switching from one drug to another. With a disease as multifactional as urticaria it is not possible to make overall dogmatic statements about which drug is best or in what dosage. The newer antihistamines can be assessed either clinically or more conveniently by studying weals provoked by intradermal injections of histamine or codeine.[53] Not surprisingly the former tend to give rather more clear-cut results as codeine liberates other mediators. UVB helps some patients. Paradoxically, aspirin and more preferably indomethacin, both well known for their ability to cause sometimes severe exacerbations of urticaria, can, if combined with an H1 blocker, provide useful symptomatic relief in some patients.

Dr V.Manna (London) provided data confirming that acrivastine (Semprex) can be added to the list of antihistamines with little or no sedative effects.

Intracutaneous injection of histamine and histamine-releasing agents to induce a weal and flare reaction can be used as a model for the study of urticaria and to assess the efficacy of antihistamines. In order to increase the sensitivity of this method a series of studies were performed by Professor R.Marks and Dr L.Lever (Cardiff) in which the following objective techniques have been used to measure the physical parameters of weal and flare. The weal and flare area were measured planimetrically using a digitizing tablet attached to a microcomputer; weal thickness was quantified using pulsed A-scan ultrasound; the degrees of erythema of the weal and flare were measured using a reflectance erythema meter; and blood flow was measured with a laser-Doppler device.[54] The results obtained from studies of a range of antihistamines have provided similar results but the effects of the drug on different parameters of the weal and flare reactions do not always parallel each other, indicating the need to perform more than one measurement for most meaningful results.

There is little published evidence that topical antihistamines are clinically effective. However, in double-blind, placebo-controlled studies using the above methods they have demonstrated that dimetindene gel (Fenistil) was not only effective in raising the itch threshold after application of histamine dilutions to abraded skin but that weal thickness, as measured by ultrasound, was also reduced (unpublished data).

Throughout the symposium it was clear that in the fairly near future an improved understanding of the mechanisms of urticaria of different types can be expected to lead on to more specific lines of treatment.

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British Journal of Dermatology (1991) 124, 109–114.

Correspondence

Topical cyclosporin and T lymphocytes in keloid scars

SIR, True keloid scars in white-skinned people nearly always occur spontaneously and have a specific anatomical distribution in the presternal area or neighbouring skin.[1] Less typical keloids can appear after minor injuries over the deltoid region (after BCG vaccination) and in the ear lobes (after ear-piercing). These scars progressively invade normal skin and remain active for many years. As yet there is no satisfactory treatment for keloid scars. The mechanisms initiating and perpetuating keloid activity are unknown, however the persistence of perivascular T lymphocytes associated with increased collagen deposition has recently been implicated in the development of abnormal scars.[2,3] Moreover, mice depleted of T cells show depressed wound healing ability, as assessed by wound breaking strength and fibroblast collagen synthesis.[4] Similar effects have been observed in rats following systemic cyclosporin A (CyA) administration.[5] Conceivably therefore, CyA might suppress abnormal scar activity. We were interested to read the report of Schulze *et al.*[6] that topical CyA in an ointment base was absorbed in adequate quantities into the deeper epidermis and upper dermis of psoriatic plaques, yet failed to affect the mononuclear cell infiltrate, although there were reductions in infiltrating neutrophils.

We have examined the influence of a recently developed topical cyclosporin A formulation[7] on keloid scar activity and lesional T lymphocytes in two Caucasian male patients aged 38 (Patient 1) and 20 years (Patient 2), with established keloid scars measuring 2·7 and 4·9 cm^2, respectively. Eight per cent w/v CyA (Sandimmun®, Sandoz) containing the penetration enhancers 18% propylene glycol (Sigma, Poole, Dorset, U.K.) and 2% azone (Nelson Research, Irvine, CA, U.S.A.) was applied twice daily by dropper to occluded scars for 4 weeks. The daily amount of CyA applied to cover each scar was 25 mg (Patient 1) or 50 mg (Patient 2). A 3-mm punch biopsy was taken near the active scar edge 2 weeks prior to starting treatment and at the end of the study. The streptavidin–biotin peroxidase staining technique[8] was employed to demonstrate leucocyte phenotypes and activation markers, using primary mouse anti-human monoclonals directed against CD1 (Langerhans cells), CD3 (pan T cells), CD4 (T-helper cells), CD8 (T-cytotoxic/suppressor cells), CD25 (interleukin 2 receptor; IL-2R) and HLA-DR.

Before treatment, biopsies from both patients revealed that T-lymphocyte distribution was focal in the epidermis and diffuse in the upper dermis; approximately 25–30% of these cells were IL-2R positive. Following 4 weeks of CyA application, there were uniform reductions in T-cell numbers (both CD4$^+$ and CD8$^+$) and in HLA-DR expression within the lesions of both patients. IL-2R$^+$ cells were also reduced, although more markedly in Patient 2 (Fig. 1). No obvious change in the external appearance of the scars was noted over the 4-week study. This is not surprising as, even if fibroblast activity was completely inhibited, it would take months or even years for the bulk of collagen to be removed. If, however, T lymphocytes prove to be an essential link in perpetuating scar activity, these observations suggest that early application of CyA in an excipient which maximizes percutaneous drug absorption might be of value in limiting lymphocyte and consequently fibroblast activity in abnormal scar formation.

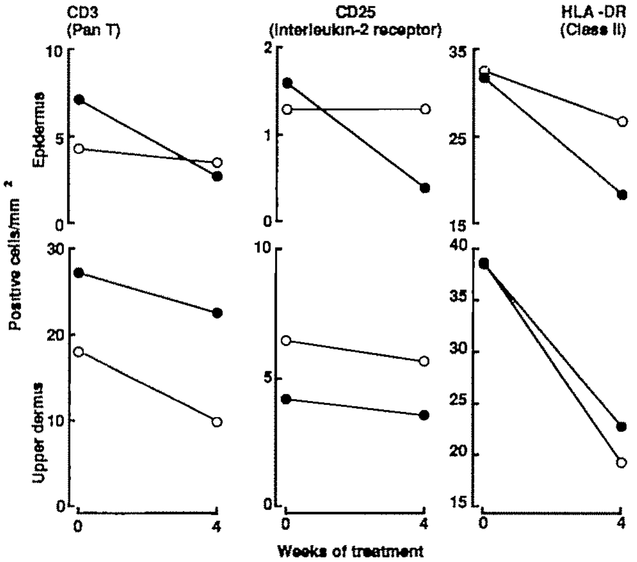

Figure 1. The influence of topical cyclosporin A on T cells, interleukin 2 receptor and HLA-DR expression within keloid scars. ○, Patient 1; ●, Patient 2.

Immunopathology Laboratory, JANET I.DUNCAN
Department of Pathology; A.W.THOMSON
**Department of Surgery,* *I.F.K.MUIR
University of Aberdeen Medical School,
Foresterhill, Aberdeen AB9 2ZD, U.K.

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Topical (1%) solution of griseofulvin in the treatment of tinea corporis

SIR, The efficacy of a new formulation of griseofulvin as a topical solution in the treatment of tinea corporis was investigated in a double-blind randomized placebo-controlled clinical study at the Manila Doctors Hospital, Manila, Philippines. The solution comprises griseofulvin in the following mixture of solvents: guciacol glyceryl ether, benzyl alcohol, acetone and isopropyl alcohol. The proportions of these solvents allow the active product to be transferred to the less volatile solvents, whilst those which are more volative evaporate on contact with the skin. Griseofulvin can therefore be carried across the dermal surface. This patented trans-phase delivery system (U.S. patent number: 4820724—11.4.89) is represented diagramatically in Figure 1.

Fifty-three patients with tinea corporis (33 male, 20 female; age range 19–70 years, mean 30 years) were recruited. Twenty-seven were randomly chosen to receive the placebo solution prepared from the excipients of the active preparation. The remaining patients were treated with the griseofulvin solution (1%). The treatment was applied daily to each affected area at a dose of 0·15 ml administered from a metered dose spray of 0·05 ml (equivalent to 0·5 mg of griseofulvin) for a period of 14 days. The mean total dose of griseofulvin applied over 14 days was 92 mg (range 40–185 mg, $n=26$).

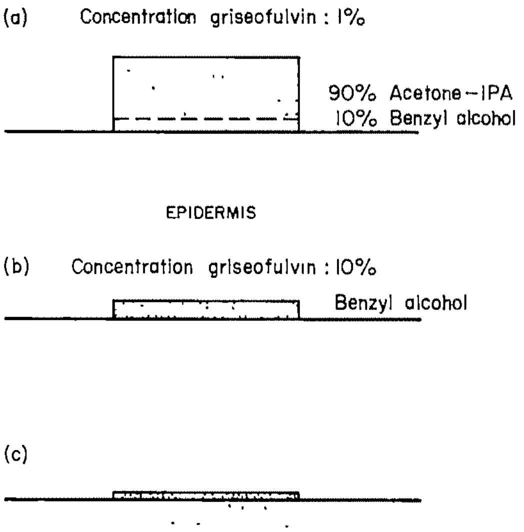

Figure 1. Diagramatic representation of the trans-phase delivery system of griseofulvin solution (1%) (a) At the time of application Griseofulvin solution is applied to skin (for ease of diagrammatic representation, a smooth idealized skin surface is used). (b) 10–15 s post-application. The more volatile solvents readily evaporate. When 0·1 ml of solution is applied it evaporates within 10–15 s leaving a thin film of the less volatile solvent (benzyl alcohol) which at skin-surface temperature can hold up to 30% griseofulvin. (c) Five min post-application. Benzyl alcohol carrying the concentrated levels of griseofulvin is absorbed into the keratinized layer of the dermis within 5–10 min as judged by the disappearance of its characteristic odour and of the residual thin film left after the volatile solvents evaporate.

The initial diagnosis of tinea corporis was made clinically and provisionally confirmed by detection of hyphae and a potassium hydroxide wet mount. The final diagnosis was made from mycological culture. Any patient in whom the culture at the beginning of treatment was subsequently shown to be negative was withdrawn from the study. Additional exclusion criteria were any infection/superinfection of the skin requiring additional treatment, treatment with systemic antifungals during the 30 days preceding entry to the trial, systemic steroids and/or topical antifungals in the preceding 14 days and topical steroids during the previous 7 days.

Symptoms were assessed on entry and following 7 and 14 days of treatment. Mycological cultures were undertaken on days 0, 7 and 14. The occurrence of side-effects was monitored at each clinical visit. The overall response to treatment was graded at day 14 as follows. (i) Complete cure: mycological culture and microscopy negative. No residual signs and symptoms. (ii) Mycological cure: mycological culture and microscopy negative. Residual erythema and/or desquamation present but no other clinical signs and symptoms. (iii) Improvement: residual signs and symptoms and/or pathogen detectable but clinical signs and symptoms improved. (iv) Failure: no significant response to therapy or exacerbation.

The fungal species most commonly identified was *Trychophyton rubrum* (68%), whilst in 28% of patients *T. mentagrophytes* was the causative agent. In two patients (4%) the fungal species was *Microsporum gypseum*. The duration of disease ranged from less than 1 week to 156 weeks (median 10 weeks). The placebo and active treatment groups were homogeneous with regard to demographic factors and clinical form.

At day 14 the distribution of severity scores for the most commonly occurring symptoms of scaling, papulation and erythema (present in all patients on entry) and pruritus that occurred in 83% of patients were significantly lower in those treated with griseofulvin solution compared to those receiving placebo ($P<0{\cdot}001$, Mann–Whitney U-test). In the overall assessment of patient response at day 14, 19 (73%) of patients in the active treatment group were graded as having experienced complete cure, as compared to two of the placebo-treated patients ($P=0{\cdot}0001$, χ^2 test). Of the patients receiving placebo, 63% failed to respond to treatment (Table 1).

No adverse events were reported during the study period, but side-effects of burning/stinging following application of trial

Table 1. Summary of response to treatment on day 14

Treatment	n	Complete cure	Mycological cure	Improvement	Failure to respond
0·15 ml griseofulvin solution	26	19 (73·1)	2 (7 7)	5 (19·2)	0
0·15 ml placebo solution	27	2 (7·4)	1 (3 7)	7 (25·9)	17 (63 0)
Total	53	21 (39·6)	3 (5·7)	12 (22·6)	17 (32 1)

medication were reported by 66% of patients (16 in the active treatment group, 19 in the placebo group). However, in all cases these side-effects were transient lasting from a few seconds to 5 min post-application of solution and abating spontaneously. Three patients (two on placebo solution, one on griseofulvin solution) reported local irritation/redness and one patient, of the placebo treatment group, experienced dryness of the skin. No patients discontinued treatment or withdrew from the trial as a result of these side-effects. None of the side-effects frequently reported during high dose oral griseofulvin therapy[1] were observed. This is as expected, as the mean dose of griseofulvin applied during the study period (92 mg) is equivalent to a daily dose of 6·60 mg, i.e. 75–150-fold less than that which is commonly required orally.

The results show that this formulation of topical griseofulvin is both safe and effective in the treatment of tinea corporis. Further studies are currently in progress to assess the efficacy of this preparation in the treatment of tinea pedis and tinea cruris.

Manila Doctors Hospital,
Ermita,
Manila,
The Philippines;
**Tillotts Pharma*
Henlow,
Beds SG6 6DS, U.K.

E.N.MACASAET
PAULINE PERT*

Reference

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ADONIS 000709639100021P

Very low dose acyclovir can be effective as prophylaxis for post-herpetic erythema multiforme

SIR, A number of reports have documented the efficacy of prophylactic oral acyclovir in suppressing recurrent erythema multiforme (EM) associated with herpes simplex virus (HSV) infection.[1–4] To be effective a dose of 200–800 mg twice daily has generally been required; however, acyclovir is an expensive drug especially when used in an extended prophylactic manner. We report a case in which only 200 mg once daily was required for effective disease suppression.

From 1985 to 1988 a 37-year-old woman suffered approximately four episodes a year of severe EM associated with HSV infection necessitating hospital admission on several occasions. Each attack lasted 3–4 weeks and resulted in considerable loss of time from work. Acyclovir 200 mg five times a day was started at the onset of prodromal herpetic symptoms and lessened the severity of EM but did not suppress the attacks completely. Prophylactic oral acyclovir was then instigated at a dose of 400 mg twice daily and continued for 6 months. No episodes of EM occurred whilst on treatment but 6 weeks after stopping the prophylaxis an episode of post-herpetic EM developed. Treatment with acyclovir was restarted at a dose of 200 mg twice daily for 6 months followed by 200 mg once daily for a further 6 months. Again complete suppression of attacks was achieved but 2 weeks after cessation of acyclovir a further attack of EM associated with HSV infection occurred. The patient is now receiving 200 mg once daily and no new episodes of HSV infection or EM have occurred over the last 5 months. No adverse effects have been encountered either clinically, haematologically or biochemically throughout acyclovir treatment. It is our intention to continue prophylaxis for a further 2-year period before attempting to discontinue acyclovir.

This case demonstrates that very low-dose acyclovir may be both safe and effective in suppressing HSV infection and associated EM. Even lower doses than those employed in this case might be equally effective because, when used prophylactically, the drug is available at the earliest moment of possible viral reactivation. It is appropriate to stop prophylaxis intermittently to determine whether the natural history of the disease has 'burnt out', but at the low dose of 200 mg once daily long-term prophylaxis is an economic and practical proposition. We would suggest that whenever acyclovir is used as prophylaxis for post-herpetic EM the dose should be titrated down according to clinical response, recognizing that the required dose may be far less than that used for the treatment of acute HSV infections.

Department of Dermatology,
University of Glasgow and
Western Infirmary, Glasgow
G11 6NT, U.K.

R.E.A.WILLIAMS
ROSEMARY LEVER

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ADONIS 0007096391000224

Immunopathological studies of Sweet's syndrome

SIR, The pathogenesis of Sweet's syndrome remains unknown, and though thought to be a hypersensitivity vasculitis, it is now regarded as an autoimmune disease.[1,2] We have examined the immunohistology of a typical case of Sweet's syndrome that occurred in a 44-year-old woman. The immunohistology of the inflammatory dermal infiltrate seen in this patient is summarized in Table 1. Our findings are similar to those described by Aiba and Tagami[3] in Schamberg's disease, formerly considered along with Sweet's syndrome to be a specific chronic vasculitis. These authors remarked that cellular immune reactions taking place in the lesional skin may play an important role in the pathomechanism of Schamberg's disease. The absence of a vasculitis in Sweet's syndrome supports a cellular immune reaction as the pathogenetic

Table 1. Immunophenotype characterization

Antibodies	Predominant immunoreactivity	*	Location
CD1	Langerhans cells	+	Epidermis, dermis.
CD2	T cells (sheep erythrocytic rosette receptor)	++	Dermis, epidermis (focal)
CD4	Helper/inducer T-cell subset	++	Dermis, epidermis (focal)
CD8	Suppressor/cytotoxic T-cell subset, NK cells	+	Dermis
CD11b	Large granular lymphocytes, monocytes and granulocytes	++	Dermis, epidermis (focal)
CD15	Mature granulocytes, monocytes	+	Dermis
CD25	Interleukin 2 receptor	+	Dermis
CD30	Sternberg–Reed cell associated Ag	−	
CD35	C3b receptor	++	Dermis, epidermis (isolated cells)
CD45	Leucocyte common antigen	+++	Dermis, epidermis (focal)
CD45R	B lymphocytes	−	
UCHL-1	T lymphocytes, granulocytes	++	Dermis, epidermis (isolated cells)
Leu 7	Large granular lymphocytes (including NK cells)	−	
HLA-DR	Dendritic cells, Langerhans cells, activated T lymphocytes	++	Dermis, epidermis (isolated cells)
Ki-67	Proliferating cells	+	Dermis

*Estimated number of staining cells, −, +, ++, +++.

mechanism. Lymphokines secreted by helper T lymphocytes with subsequent chemotaxis of neutrophils and macrophages[4] may be responsible for the histopathology seen in this syndrome.

As an upper respiratory tract infection is often associated with Sweet's syndrome, it could be that the factor responsible for the cellular immune response is a cross-reaction with tissue antigens. The frequent association with other autoimmune diseases[5] and lymphoid and myeloproliferative disorders[6] suggest that patients with Sweet's syndrome have an underlying immunological disorder, possibly a defect in suppressor T lymphocytes.

Department of Pathology,
Granada University School of Medicine,
Granada,
Spain

T.Alvaro
R.Garcia del Moral
M.Gomez-Morales
J.Aneiros
F.O'Valle

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ADONIS 000709639100023H

Phototumorigenicity of 6-methylangelicin derivatives

Sir, We were interested to read the article by Cristofolini and co-workers[1] who investigated 6-methylangelicin derivatives that may have photochemotherapeutic potential.

One of the aims of developing novel photochemotherapeutic agents is to reduce adverse effects associated with currently used compounds such as 8-methoxypsoralen (8-MOP). 8-MOP is photomutagenic in many biological systems,[2] phototumorigenic in mouse skin[3] and may increase the risk of developing non-melanoma skin cancer in PUVA patients.[4] The authors presented data on the comparative photomutagenicity of 6,4,4′-trimethylangelicin (6,4,4′-TMA) and 8-MOP in three recognized *in vitro* genotoxicity assays using bacterial, yeast and cultured mammalian cells Expressed in terms of cell survival, 6,4,4′-TMA photomutagenic activity equalled that of 8-MOP in mammalian cells but was less than 8-MOP in the other two assays.

In an earlier report to the European Society for Photobiology in 1987, Santamaria and colleagues[5] suggested that 6,4,4′-TMA showed considerably higher photomutagenic activity than 8-MOP in three different *in vitro* genotoxic assays. Significantly, it was also reported that 6,4,4′-TMA was phototumorigenic in mice exposed to 365 nm radiation. It is essential, when evaluating new photochemotherapeutic agents, to consider potential genotoxic and tumorigenic risks. Cristofolini and co-workers have clearly demonstrated that

6,4,4′-TMA has photomutagenic activity but have omitted to relate these findings to the reported phototumorigenic activity of this compound.

Photobiology Unit, N.K.GIBBS
Department of Dermatology, B.E.JOHNSON
Ninewells Hospital and Medical School, J.FERGUSON
Dundee DD1 9SY, U.K.

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ADONIS 000709639100024U

Local anaesthesia with ethyl chloride freezing: problems despite proper application

SIR, After a century of use of ethyl chloride for general anaesthesia and hundreds of deaths due to its narrow safety margin,[1] the application of ethyl chloride is now restricted to local anaesthesia for punch biopsies,[2] curettage of superficial lesions, the piercing of ears,[3] the freezing of lesions such as granuloma annulare and for sports injuries.[4] Caution is, however, required because ethyl chloride is highly inflammable and a potent inhalation anaesthetic.[5] Appropriate room ventilation is essential, and its use is prohibited where there may be sparks or an open flame. Prolonged spraying causes chemical 'frost bites', ulceration of the skin and muscle damage.[3,5] Freezing also distorts the histological structure of biopsy specimens.[2] Nonetheless, ethyl chloride is a very effective, convenient and therefore a popular way of delivering short-term local anaesthesia and has, in general, proven to be safe if properly administered.

This chemical agent can also elicit unexpected allergic reactions. We have recently seen[6] a 66-year-old female who suffered from generalized mollusca contagiosa. Removal of the lesions by curettage after ethyl chloride freezing caused peculiar patterns of eczematous dermatitis in the skin surrounding the treated areas. These were interpreted as a contact dermatitis due to the disinfectants, antibiotics or the tape used. A diagnosis of contact eczema was confirmed by histology, and open patch testing with ethyl chloride showed an allergic reaction (Fig. 1) which was also seen with related propellants.

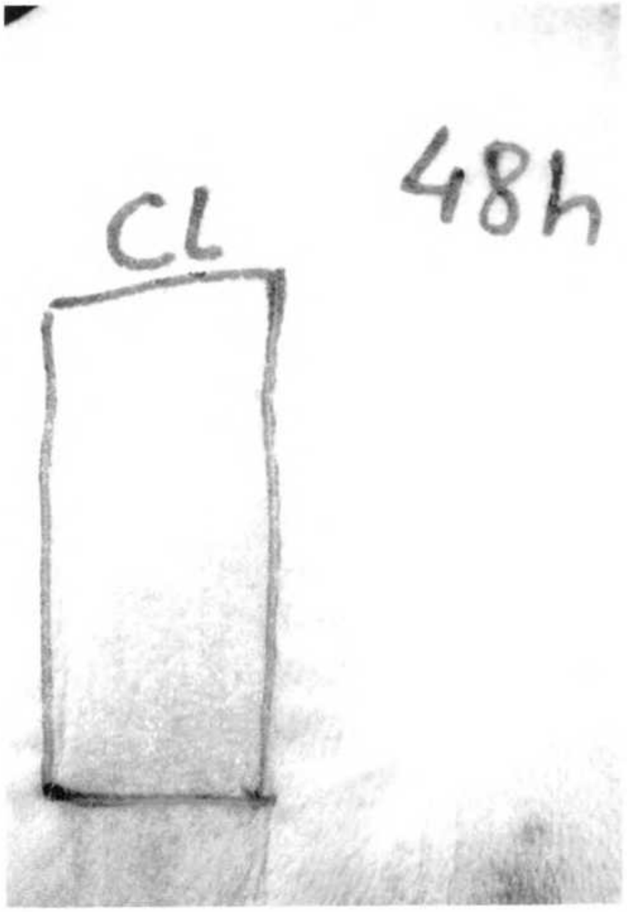

Figure 1. Positive patch-test reaction (erythema and vesicles) 48 h after exposure to ethyl chloride (Cl) and negative control (48 h) using a chemically non-related propellant.

The patient reported that she had never tolerated deodorants, female hygiene sprays and medical 'wound sprays' which contain these propellants.[7] Three similar cases of allergy to ethyl chloride have since been brought to our attention.

Proper use of ethyl chloride for short-term local anaesthesia is assumed to be safe. Considering its widespread use in dermatology, there are few serious adverse effects. Allergic reactions, as dose-independent phenomena, occur not infrequently but are usually misinterpreted as toxic reactions or allergy to the other topical agents. In addition, the similar chemical structure and properties of ethyl chloride with the liquefied gas propellants[7] may result in a group sensitivity.[8] These halogenated propellants are widely used but are now gradually being replaced.

Department of Dermatology, I W.ABERER
University of Vienna,
Vienna, Austria

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ADONIS 000709639100026M

Vascular involvement and toxoplasma infection

SIR, In 1979 a case was reported in this journal under the title of 'Dermatomyositis-like syndrome due to toxoplasma'.[1] This was a 44-year-old female with a history of articular pain, myalgias and wasting of muscles, particularly of the deltoids and biceps. She had violaceous nummular patches with fine pityriasiform scaling on the flexor surface of the forearm and on the palms. An initial diagnosis of dermatomyositis was made but later investigations confirmed that she had infection with toxoplasmosis. A muscle biopsy revealed numerous toxoplasms, some free and some in muscular fibrocytes but mostly intracytoplasmic in the macrophages and in polymorphonuclear cells. She was treated with seven successive 1-month cycles of treatment with pyrimethamine (25 mg) and sulphamethoxypyridine (0·5 g) every second day.

In March 1986 the patient was investigated for angina. At the age of 54 in April 1987 she was again submitted to our clinic because her cutaneous and muscular symptoms had reappeared. She was found to have stenoses of the left subclavian artery and the left dorsalis pedis artery. A Doppler scan and arteriography confirmed the presence of these stenoses. Laboratory investigations carried out gave positive serological tests for toxoplasmosis.

The patient had four successive cycles of treatment with pyrimethamine and sulphamethoxypyridine at the same dosages as used previously. The symptoms of myalgia and her angina disappeared and the electrocardiogram became normal after the first cycle of therapy. Serological tests for toxoplasmosis became negative after three cycles of therapy. To date the vascular stenoses have not got worse and there has been no further vascular involvement. We present this case again for two reasons: firstly, that toxoplasmosis recurred 3 years after apparent clinical recovery and normal laboratory tests. Secondly, there was a relapse of disease in this patient associated with multiple arterial stenosis. Further treatment in our patient for toxoplasmosis has alleviated her angina and there has been no further progression of the vascular stenosis. We presume that the toxoplasma infection caused this vascular involvement though we have not found a similar case in the medical literature.

Department of 1st Clinica Medica
La Sapienza University
Rome
Italy

G.CARMENINI
A.TOTO
S.MARTUSCIELLO
B.VALIANTE
A.MAGRINO
F.MELONI

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ADONIS 000709639100027Z

British Journal of Dermatology (1991) **124**, 115. ADONIS 000709639100028E

Book Reviews

Trattato Di Dermatologia. Edited by FERDINANDO SERRI (1986). 5 Volumes. Padua: Piccin. ISBN 88-299-0689-1. Price £562.

For readers of the *British Journal of Dermatology* whose knowledge of Italian has progressed beyond an appreciation of Pavarotti, Professor Serri has assembled the entire dermatological orchestra of Italy with some invited foreign guests to produce a major opus comprising five loose-leaf volumes and a separate index volume. It will occupy 37 cm of your library shelf, and a stout shelf is recommended!

The whole of Volume 1 is devoted to basic sciences, epidemiology, genetics and histopathology. Volume 2 concentrates on infectious diseases, contact dermatitis and atopic dermatitis. Volume 3 moves from urticaria through precancerous dermatoses, psoriasis, oral medicine and hirsutism. Volume 4 includes skin tumours, genodermatoses, connective tissue disease and vasculitis, and Volume 5 begins with a section on lymphoma, moves on to a large section on dermatological therapy, venereology, and finishes with a chapter on AIDS-related problems. We thus have a comprehensive Italian text comparable in content with Rook or Fitzpatrick. High quality glossy paper is used and there are numerous colour illustrations.

As one would expect from Professor Serri, the section devoted to basic sciences, including embryology, is extensive, well-written and comprehensive. I was, however, disappointed with relatively brief cover of melanoma, which merited only 18 pages and nine references, all of these in English. The illustrations in this chapter, however, are excellent. In contrast, the next chapter, on 'connective tissue tumours', although less well illustrated, was accompanied by 191 references. In some chapters there is a tendency to refer to review articles rather than the original work and this is seen in the chapter on tropical dermatology. The numbering of the pages makes it a little difficult to find one's way initially, as each chapter begins on page 1, but once this problem is overcome this is a very pleasant book to study and it will undoubtedly find friends in the Italian-speaking community. The price is however daunting, even by current comparative standards with larger textbooks.

RONA M.MACKIE

The Skin in Rheumatic Disease. C.R.LOVELL, P.J.MADDISON, G.V.CAMPION (1990). London: Chapman and Hall Medical. Pp. 148. 129 black and white, 10 colour illustrations. ISBN No. 0-442-29000-6. Price £19.95.

This book states its aim as giving a succinct account of cutaneous manifestations of the major connective tissue diseases, and is directed at dermatologists and rheumatologists, with general physicians and general practitioners also benefiting. Written by clinicians active in the field, and showing many signs that this is the case, the book achieves that aim: it is succinct, accurate and easy to read. A lot of information is conveyed, with the major diseases given a chapter each, covering aetiology, clinical features and therapy. The other chapters are devoted to clinical topics—vasculitis and infections to name but two—with a helpful contribution on drug reactions containing a simplified table that indicates the complexity of this area. I also like the chapter on diagnostic problems, and though this, like the rest of the book, is aimed at the skin, a small section on the joints would have been of value to non-rheumatologists.

The book is liberally illustrated, with many fine photographs, though not all the black and white figures are sufficiently clear. There is a tendency to put in some photographs because they are good examples, e.g. four figures of the very rare multicentric reticulo-histiocytosis. In addition, I found some of the histological sections too small to be of value. There is also the occasional missed opportunity—an excellent diagram comparing morphoea and lichen sclerosus et atrophicus would have exactly complemented a striking pair of clinical and histological figures showing the same thing, but is separated by several pages.

However, these are small criticisms overall, and the book does 'fill an important gap'. At £19.95 it is reasonably priced, and would be a welcome addition to our library, particularly for the middle-grade staff.

M.GOODFIELD

British Journal of Dermatology (1991) 124, 116.

News and Notices

The Institute of Dermatology, United Medical and Dental Schools, University of London

The respective titles of Professor of Experimental Dermatology and Professor of Cell Pathology have been conferred on Drs R.D.R.Camp and R.A.J.Eady of the Institute of Dermatology, United Medical and Dental Schools, University of London.

5th International Symposium on Psoriasis and Psoriasis–Arthritis 9–15 March 1992, Jerusalem

The 5th International Symposium on Psoriasis and Psoriasis–Arthritis will be held in Jerusalem on 9–15 March 1992. For further information contact: Mr Yahalom, Health Vacation Center Ltd, P.O.Box 99, Ramat Hasharon 47100, Israel.

2nd Congress of the European Academy of Dermatology and Venereology (EADV) 10–13 October 1991, Athens

The 2nd Congress of the European Academy of Dermatology and Venereology will be held in Athens on 10–13 October 1991. The Congress will be organized by the European Academy of Dermatology and Venereology in co-operation with the Department of Dermatology of the University of Athens. For further information contact: Congress Organizers and Secretariat, Triaena Congress, 24 Harilaou Trikoupi Street, Athens 106 79, Greece.

British Society for Dermatopathology
Guildford, 8 February 1991

The British Society for Dermatopathology is holding its Winter Meeting on 8 February at the Postgraduate Medical Centre, the Royal Surrey County Hospital, Guildford. The main theme of the meeting will be 'Disorders of the Pigmentary System'. There will be also a session of free papers and a slide seminar, neither of which will be confined to melanocytic problems. The registration fee is £50. For further details please contact: Dr Martin Cook, Department of Histopathology, Royal Surrey County Hospital, Guildford. Telephone number 0483 571122 extension 4257.

Workshop on Cutaneous Lymphoproliferative Disorders and Adnexal Tumours, 21 March 1991, Sheffield

A 1-day Teach-In Workshop on Cutaneous Lymphoproliferative Disorders and Adnexal Tumours will take place at Sheffield University Medical School on 21 March 1991. This has been arranged in conjunction with the British Society of Dermatopathology for the new Royal College of Pathologists postgraduate qualification in Dermatopathology. The morning session will be devoted to cutaneous lymphoproliferative disorders and the afternoon session to cutaneous adnexal tumours. For further information contact: Dr D.N.Slater, Department of Histopathology, Rotherham District General Hospital, Rotherham S60 2UD or Dr D.W.K.Cotton, Department of Pathology, Sheffield University Medical School, Sheffield S10 4LA.

71st Annual Meeting of the British Association of Dermatologists 3–6 July 1991, London

The next annual meeting of the British Association of Dermatologists will be held in London from 3–6 July 1991 under the Presidency of Dr Harvey Baker. Dr Michael Klaber will be acting as Local Secretary. The scientific meeting will be held at Kensington Town Hall and on the morning of Saturday 6 July there will be a clinical meeting at the Royal London Hospital. There will be a short clinicopathological conference (CPC) on Thursday morning, 4 July after the AGM, and suitable case reports are invited.

Abstracts of papers and papers are invited for consideration by the Selection Committee and those accepted as papers will be allotted 10 minutes for presentation and time for discussion. Abstracts of papers, posters and CPC case reports should be submitted on the official forms which are available from: Mrs R. Barton, Conference Manager, British Association of Dermatologists, 3 St Andrew's Place, London NW1 4LB. The closing date for receipt of complete abstract forms is Friday 11 January 1991. Clinical summaries are invited for patients to be demonstrated at the clinical meeting on Saturday 6 July. The closing date for receipt of summaries is Friday 25 January 1991.

Notice to Contributors

The *British Journal of Dermatology* publishes original articles on all aspects of the normal biology, and of the pathology of the skin. Originally the Journal, founded in 1888, was devoted almost exclusively to the interests of the dermatologist in clinical practice. However, the rapid development, during the past twenty years, of research on the physiology and experimental pathology of the skin has been reflected in the contents of the Journal, which now provides a vehicle for the publication of both experimental and clinical research and serves equally the laboratory worker and the clinician. To bridge the gap between laboratory and clinic the Journal publishes signed editorials reviewing, primarily for the benefit of the clinician, advances in laboratory research, and also reviews of recent advances in such aspects of dermatology as contact dermatitis, therapeutics and drug reactions. Other regular features include book reviews, correspondence and society proceedings. The Journal is the official organ of the British Association of Dermatologists and also of the Netherlands Association for Dermatology and Venereology, but it attracts contributions from all countries in which sound research is carried out, and its circulation is equally international.

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Manuscripts. Three copies of all material for publication should be sent to the Editor, Professor S. S. Bleehen, Dermatology Department, Royal Hallamshire Hospital, Sheffield S10 2JF. Manuscripts should be typewritten on one side of the paper only, with a wide margin, be double spaced, and bear the title of the paper, name and address of each author, together with the name of the hospital, laboratory or institution where the work has been carried out. The name and full postal address of the author who will be responsible for reading the proofs should be given on the first page. The author should keep a carbon copy of his manuscript. The Editorial Board reserves the right to make literary corrections.

Illustrations should be referred to in the text as 'Figs', and be given Arabic numbers, and should be marked on the back with the name(s) of the author(s) and the title of the paper. Where there is any possible doubt as to the orientation of an illustration, the top should be marked with an arrow. Each figure should bear a reference number corresponding to a similar number in the text. Photographs and photomicrographs should be *unmounted* glossy prints and should not be retouched. Colour illustrations will be accepted when found necessary by the Editor, although the author will be expected to bear the cost. Diagrams should be on separate sheets: they should be drawn with black ink on white paper or feint-ruled graph paper and should be about twice the size of the final reproduction. Lines should be of sufficient thickness to stand reduction. Each illustration should be accompanied by a legend clearly describing it: these should be grouped on a separate sheet of paper.

There should be as few tables as possible and these should include only essential data: they should be typewritten on separate sheets and should be given Arabic numbers.

Abbreviations and units. Full names with uncommon abbreviations must be given with the first mention: new abbreviations should be coined only for unwieldy names and should not be used at all unless the names occur frequently. In the title abbreviations should be identified, in the introduction and discussion they should be used sparingly. Abbreviations of units should conform with those shown below.

gram(s)	g
kilogram(s)	kg
milligram(s) (10^{-3}g)	mg
microgram(s) (10^{-6}g)	µg
nanogram(s) (10^{-9}g)	ng
picogram(s) (10^{-12}g)	pg
hours(s)	h
minute(s)	min
centimetre(s)	cm
second(s)	s
cubic millimetre(s)	mm^3
millimetre(s)	mm
millicurie(s)	mCi
milliequivalent	mmol
molar	mol l
millilitre(s)	ml
gravitational acceleration	*g*
micrometre(s)	µm
per cent	%
isotopic mass number placed as	^{131}I

These and other abbreviations and symbols should follow the recommendations of: *Units, Symbols and Abbreviations. A Guide for Biological and Medical Editors and Authors* (1988) The Royal Society of Medicine, London.

References. Only papers closely related to the author's work should be referred to; exhaustive lists should be avoided. References should be given in the Vancouver style, i.e. references appear as superscript numbers in the text, e.g. '. . . our previous report^1 and that of Smith *et al.*^{2} . . .', and are brought together numerically in the reference list at the end of the article in order of their first mention in the text. References to articles and papers should mention: (1) name(s) followed by the initials of the author(s), up to four authors: if more than four authors, include first three authors followed by *et al.*; (2) title of paper; (3) title of journal abbreviated in the standard manner; (4) year of publication; (5) volume; (6) page numbers of the article. *Thus*: Cunliffe WJ, Shuster S. The rate of sebum excretion in man. *Br J Dermatol* 1969; **81**: 697–9. References to books and monographs should include: (1) author(s) or editors(s); (2) paper (if necessary) and book titles; (3) edition, volume, etc.; (4) place; (5) publisher; (6) year; (7) page(s) referred to. *Thus*: Beare JM, Wilson Jones E. Necrobiotic disorders. In *Textbook of Dermatology* (Rook AJ, Wilkinson DS, Ebling FJ, eds), 2nd edn., Vol. 2. Oxford: Blackwell Scientific Publications, 1972; 1066.

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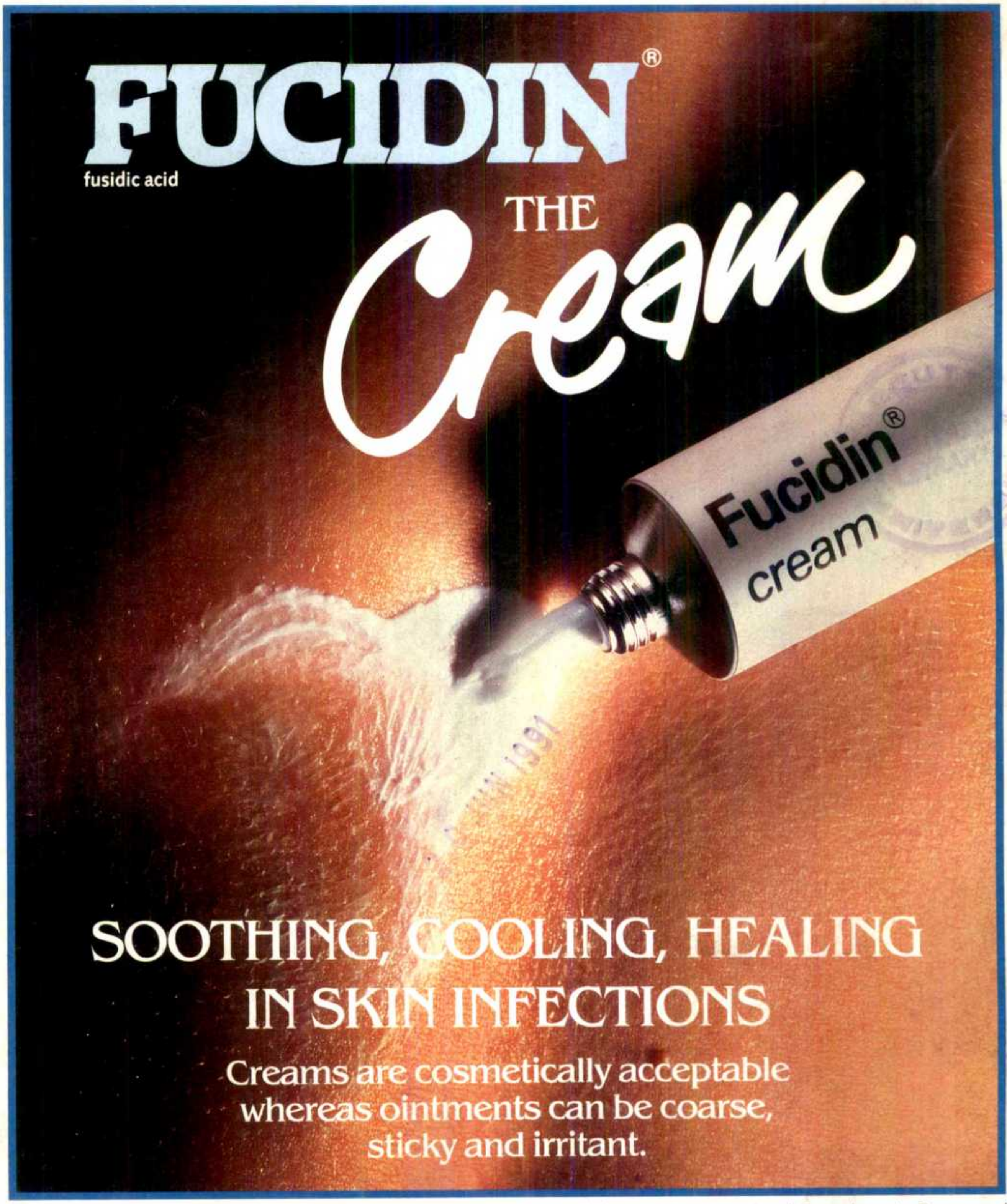
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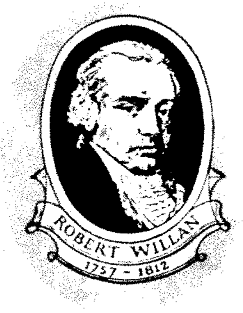

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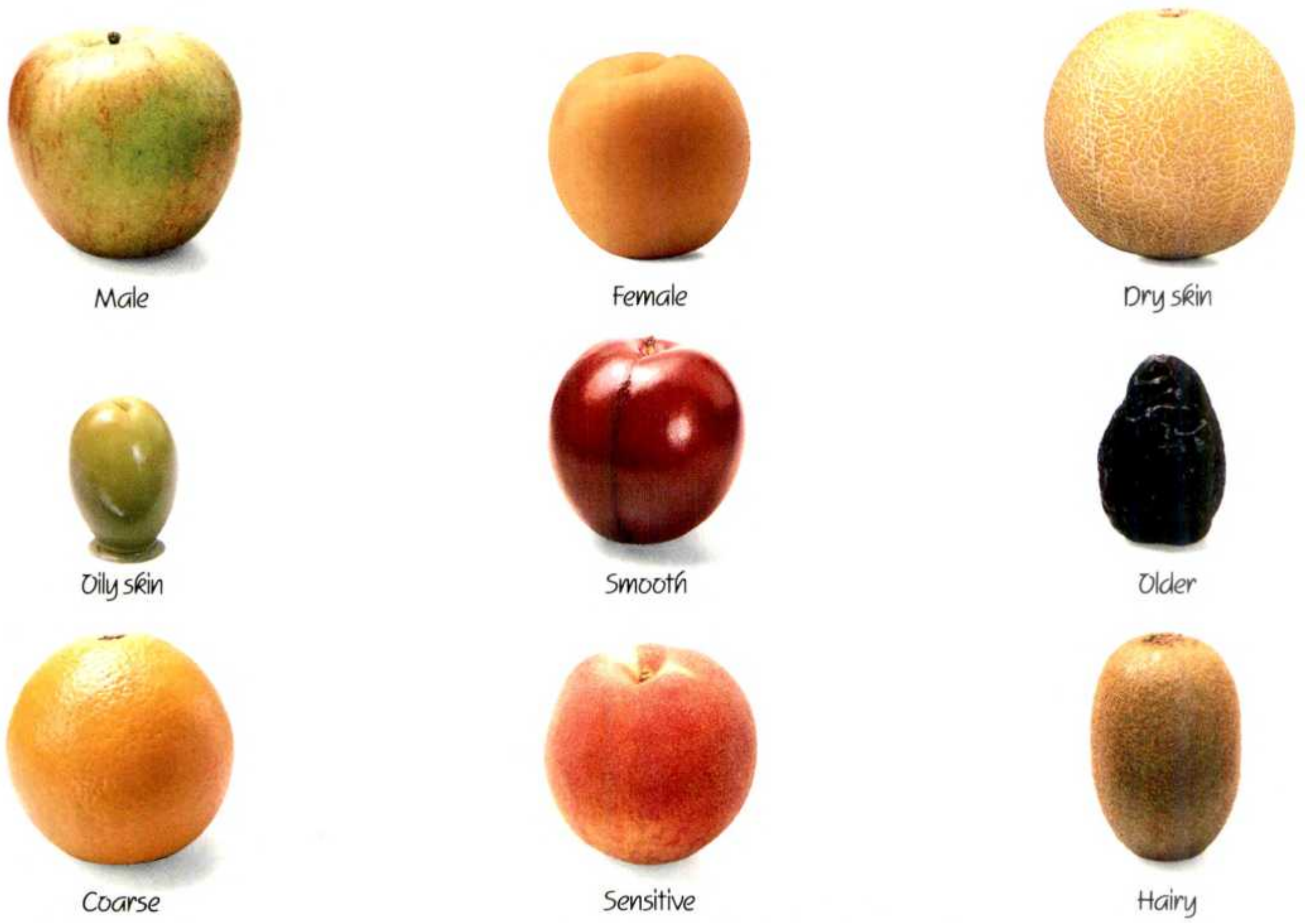

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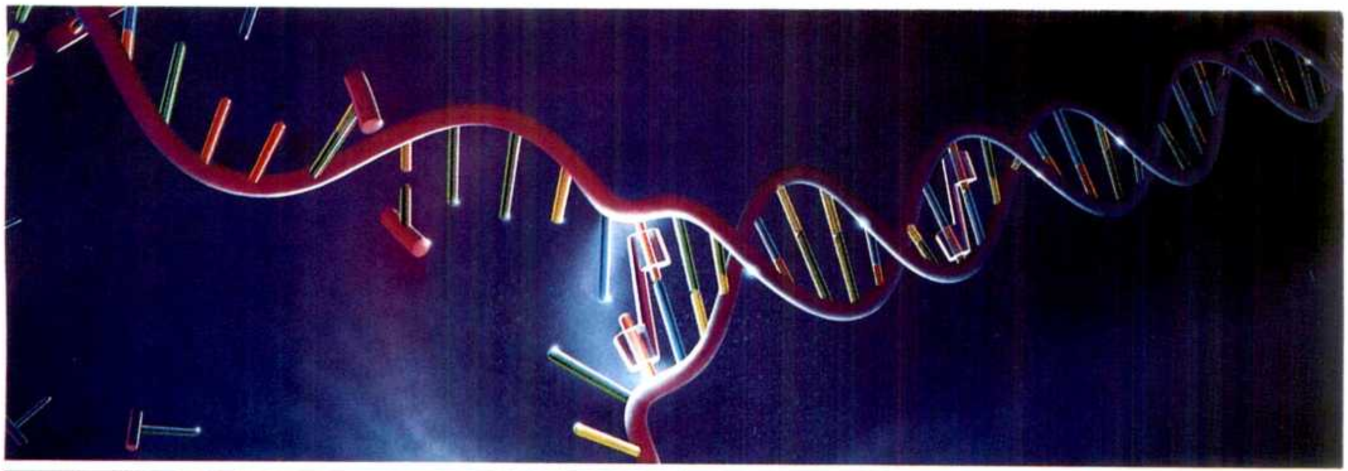

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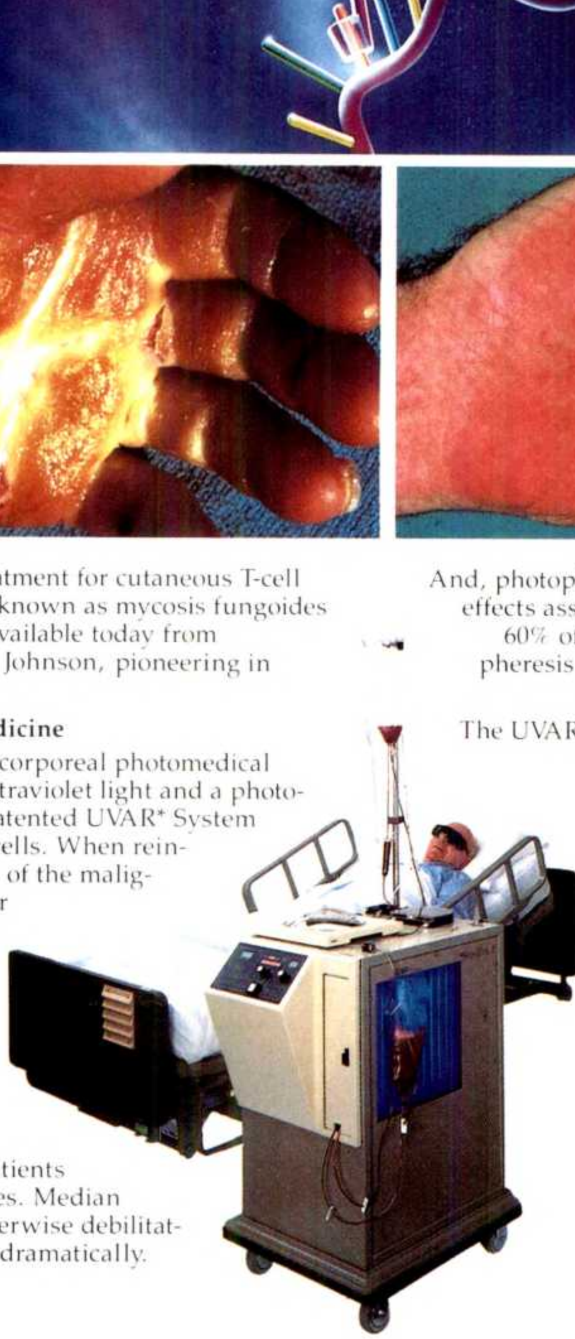

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[1] Edelson, R.L.: Light-activated Drugs. Scientific American 259(2):68-75, August 1988.

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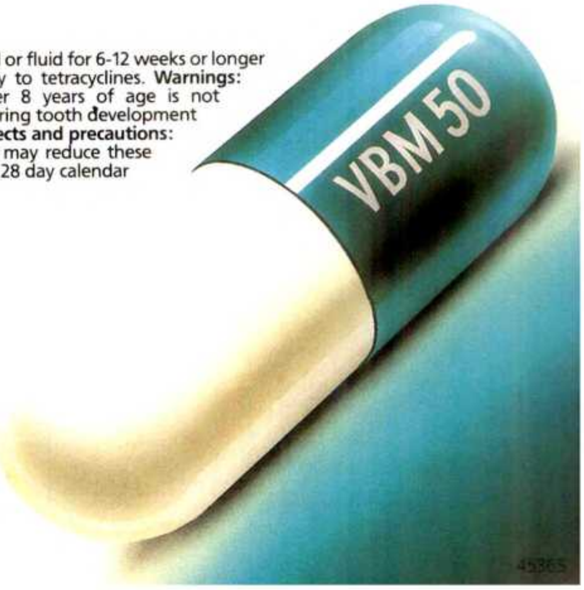
1. Harrison, P.V., (1988) Clin. Exp. Dermatol 13, 242-244



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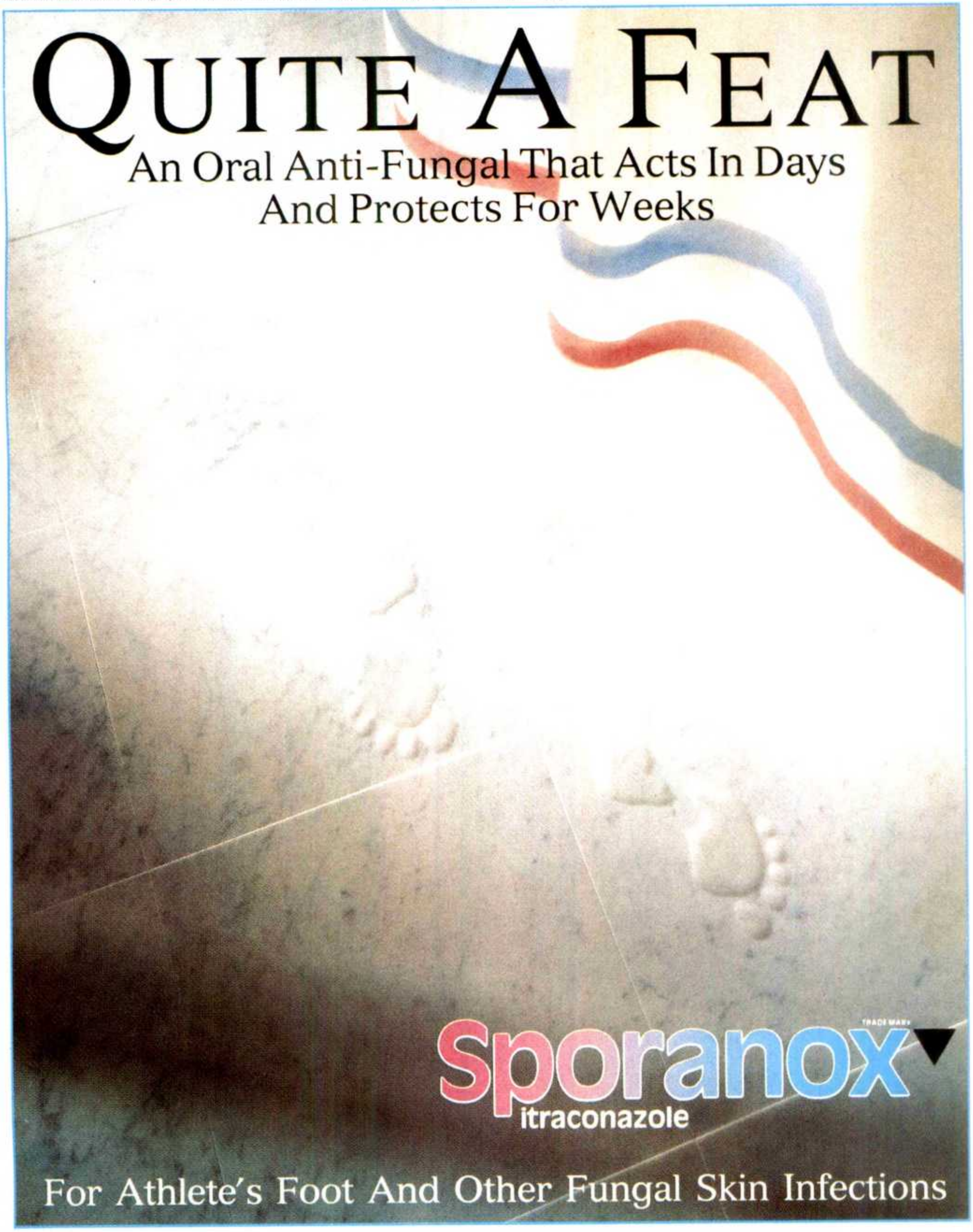
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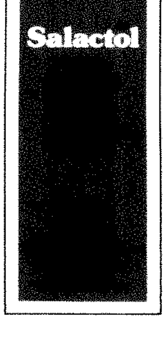

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Endothelial leucocyte adhesion molecule-1 (ELAM-1) expression in cutaneous inflammation

R.W.GROVES, M.H.ALLEN, J.N.W.N.BARKER, D.O.HASKARD* AND D.M.MACDONALD
Laboratory of Applied Dermatopathology and
**Department of Medicine (Rheumatology Unit), UMDS (Guy's Campus), Guy's Hospital, London, U.K.*

Accepted for publication 10 September 1990

Summary Endothelial leucocyte adhesion molecule-1 (ELAM-1) is a recently described endothelial surface glycoprotein which is inducible by interleukin 1 (IL-1), tumour necrosis factor-alpha (TNF-α) or bacterial lipopolysaccharide (LPS). Using an immunohistochemical technique and a monoclonal antibody (1.2B6) specific for ELAM-1 we have found marked vascular endothelial expression of ELAM-1 in many cutaneous inflammatory disorders, including allergic contact dermatitis, atopic dermatitis and psoriasis, and in dermal infiltrates associated with benign, premalignant and malignant keratinocyte proliferation. In normal skin, minimal levels of ELAM-1 expression were detected. In psoriasis, double-immunoenzyme staining studies revealed a close spatial relationship between ELAM-1 expression and neutrophil margination, suggesting a functional link. Recombinant human interferon-γ (30 μg) injected intradermally in normal adult human volunteers did not substantially upregulate ELAM-1 in contrast to its marked effect on intercellular adhesion molecule-1 (ICAM-1) expression, indicating that this cytokine is probably not involved in ELAM-1 induction *in vivo*. These results indicate that ELAM-1 is widely induced in cutaneous inflammation with a time course of expression that is longer than that observed *in vitro*.

As ELAM-1 acts as an adhesion ligand for neutrophils, and perhaps monocytes, the expression of this molecule in cutaneous lesions is likely to be an indication of the ability of vascular endothelium to recruit these cells from the circulation. Furthermore, the cytokine inducibility of ELAM-1 is indirect evidence for functional interactions between perivascular mononuclear cells, other resident cells and the blood vessel wall.

Neutrophil adherence to vascular endothelium is a crucial step in the development of an acute inflammatory reaction. Margination and diapedesis of neutrophils has been recognized for many years,[1] but only recently have the mechanisms underlying these processes begun to be understood. It is known that certain soluble inflammatory mediators such as the complement component C5a, platelet-activating factor (PAF) and leukotriene B4 (LTB4) are able to exert a chemotactic effect on neutrophils[2] and elevated levels of PAF[3] and LTB4[4] have been found in lesional psoriatic skin. Although the action of such mediators may explain the directed migration of neutrophils through the tissues, they do not fully explain the localization of neutrophils at the inflammatory focus. This phenomenon is now thought to be largely attributable to the induced expression of appropriate adhesion molecules on the surface of vascular endothelium.[5,6]

Endothelial leucocyte adhesion molecule-1 (ELAM-1) is a glycoprotein which mediates neutrophil adhesion to activated vascular endothelium. ELAM-1 was initially recognized as an inducible antigen on cytokine-activated human umbilical vein endothelial cells reactive with two monoclonal antibodies, H4-18 and H18-7.[7] Subsequent studies demonstrated that it is a single-chain molecule with a molecular weight of approximately 115 kDa.[8] ELAM-1 cDNA has been cloned and the primary sequence shows that it is a member of a new family of adhesion molecules, termed LEC-CAMs[9] (lectin, EGF and complement-related cell adhesion molecules). Other members of the family include GMP 140 and the murine MEL 14 antigen,[9] whose human equivalent is designated LAM-1.[10] ELAM-1 is regulated both in endothelial cell culture[8] and in whole skin organ culture[11] by

Correspondence: Dr D.M.MacDonald, Department of Dermatology, Guy's Hospital, St Thomas' Street, London SE1 9RT, U.K.

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interleukin 1 (IL-1), tumour necrosis factor-alpha (TNF-α) and bacterial lipopolysaccharide (LPS) but not by interferon-gamma (IFN-γ). Following cytokine stimulation, endothelial cells generate both ELAM-1 mRNA and protein (maximal at 2–4 h), although *in vitro* this decreases, even in the presence of continued stimulation, to basal or near basal levels within 24 h.[8,9]

ELAM-1 is clearly of potential importance in neutrophilic dermatoses such as pyoderma gangrenosum, leucocytoclastic vasculitis, dermatitis herpetiformis and Sweet's syndrome (acute febrile neutrophilic dermatosis). Additionally, there is evidence that it has adhesive properties for cells of monocytic lineage,[12,13] making it also of relevance to disorders with a prominent mononuclear cell infiltrate including dermatitis, psoriasis, and granulomatous disease. In order to examine whether ELAM-1 has a role in cutaneous inflammation, we have undertaken an immunohistochemical survey of a variety of cutaneous inflammatory infiltrates using an antibody to ELAM-1 raised against TNF-α activated human umbilical vein endothelial cells.[14]

Methods

Tissue

Normal skin, tumours and inflammatory dermatoses were obtained in our biopsy clinic following informed consent. Elliptical excision biopsy was performed under local anaesthesia, specimens were bisected and half was processed for conventional histological diagnosis. The second half was embedded in OCT compound, frozen in melting isopentane, and stored in liquid nitrogen until required. Acute allergic contact dermatitis tissue was obtained from patients attending our patch test clinic at varying times between 12 and 120 h and was in response to a variety of antigens, including nickel sulphate. Normal skin following the intradermal injection of 30 μg of interferon-γ was obtained as previously described.[15] The tumours and dermatoses studied are outlined in Table 1.

Table 1. Tissues studied

Normal skin	$n=5$
Acute contact allergic dermatitis ($t=12$–120 h)	$n=10$
Atopic dermatitis	$n=3$
Psoriasis	$n=5$
Virus wart	$n=5$
Bowen's disease	$n=5$
Basal cell carcinoma	$n=3$
Squamous cell carcinoma	$n=3$
Normal skin following intradermal IFN-γ	$n=4$

Table 2. Primary antibodies used

Clone	Specificity	Isotype	Reference
RR1/1	ICAM-1	IgG2	5
1.2B6	ELAM-1	IgG1	14
NP57	Neutrophil elastase	IgG1	20
EN4	Capillary endothelium	IgG1	18

Immunohistochemical technique

Frozen sections (5 μm) were air-dried for 15 min, fixed in acetone at room temperature for 15 min, washed and non-specific binding blocked with 20% normal swine serum. The primary antibodies employed are specified in Table 2. Primary antibody binding was detected using a conventional peroxidase–antiperoxidase reaction and visualized using 3,3′-diaminobenzidine (DAB) as chromogen. All secondary reagents were obtained from DAKO Ltd (High Wycombe, Bucks, U.K.). Sections were then counterstained with Mayers haematoxylin, dehydrated, cleared and mounted. Selected sections of psoriasis and dermatitis were double-stained for ELAM-1 and neutrophil elastase, a specific marker for cells of granulocyte lineage. Full details of the immunohistochemical technique used have been described elsewhere.[16] Briefly, the procedure was identical for the first antibody, ELAM-1, which was visualized with DAB; sections were then incubated with antineutrophil elastase antibody, which was detected with an alkaline phosphatase system (DAKO Ltd). Binding was visualized using Fast Blue BB salt as chromogen, providing a good contrast with the brown DAB reaction product. The sections were then counterstained, cleared and mounted as above.

Controls included omission of the primary antibody and substitution of the primary antibody by one of the same class but of irrelevant specificity.

Results

In normal skin, minimal staining for ELAM-1 was noted on dermal vascular endothelial cells (Fig. 1a), particularly those in the upper dermal vessels close to the epidermis. Vessels deep in the dermis did not express ELAM-1, nor did endothelial cells lining lymphatic channels. ICAM-1 was constitutively expressed on dermal vascular endothelial cells (Fig. 1b) as previously described,[17] in a distribution similar to EN4 (Fig. 1c).

ELAM-1 expression was strongly expressed in allergic contact dermatitis at 24 h (Fig. 2), both in terms of staining intensity and numbers of vessels labelled. At

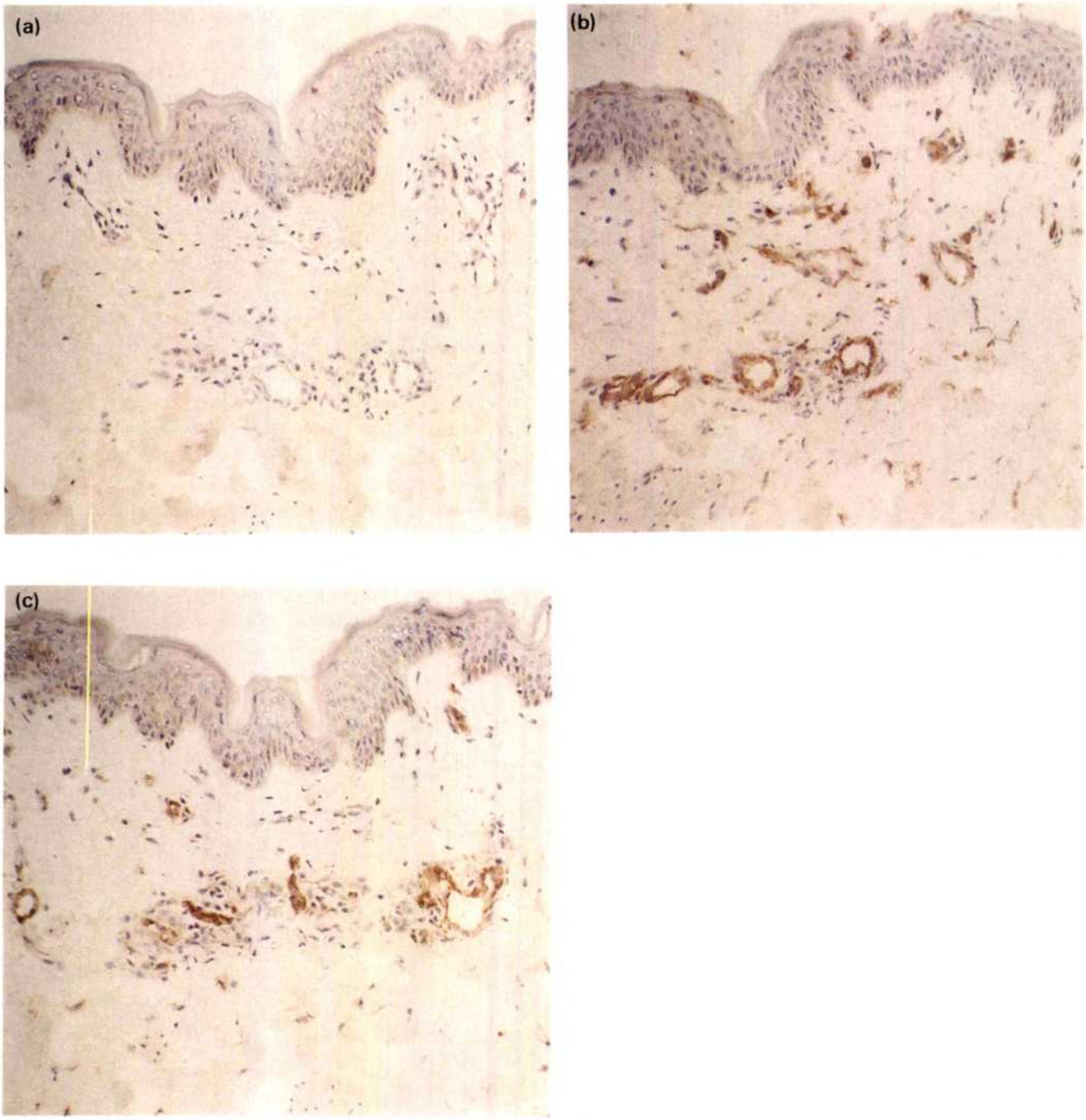

Figure 1. Serial 5-μm sections of normal skin stained for (a) ELAM-1, (b) ICAM-1, (c) EN4. Minimal staining for ELAM-1 is observed on vascular endothelium in contrast to strong expression of ICAM-1 and EN4.

this time a prominent lymphohistiocytic infiltrate was evident particularly around ELAM-1-positive blood vessels. Enhanced ELAM-1 expression persisted at all time points examined, up to 120 h. In psoriasis, ELAM-1 expression was intense and widespread, and in some areas vessels were positive in the absence of infiltrate. This was particularly so for vessels in the dermal papillae (Fig. 3). Similar results were observed in atopic dermatitis. Keratinocyte expression of ELAM-1 was not observed in any disorder studied. This contrasts with ICAM-1 where keratinocyte expression was present in allergic contact dermatitis, psoriasis (Fig. 4) and atopic dermatitis, in addition to intense staining of dermal vascular endothelium.

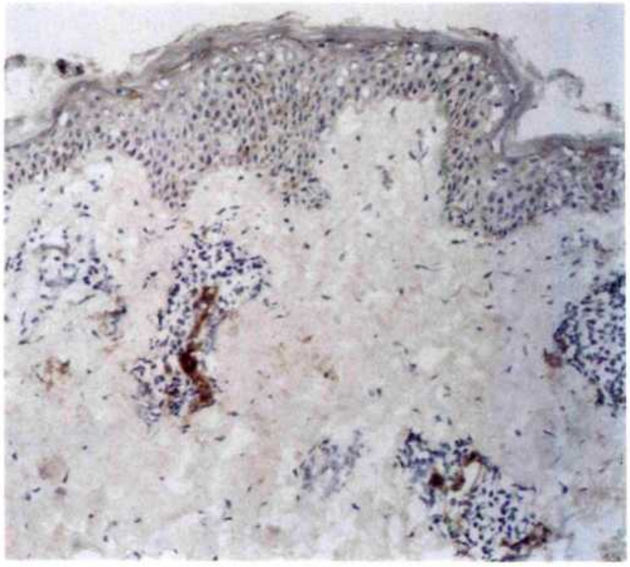

Figure 2. Allergic contact dermatitis at 24 h. There is pronounced ELAM-1 expression on endothelium within the dermal infiltrate.

In all squamo-proliferative lesions examined including viral wart, Bowens' disease (Fig. 5) and squamous cell carcinoma a prominent lymphocytic infiltrate was present around the base of the tumour. ELAM-1 was widely expressed on vascular endothelium within the infiltrate in all cases. This contrasted with the appearance in basal cell carcinoma where the infiltrate was considerably less intense. In these cases ELAM-1 expression was undetectable on vessels within the tumour stroma which were clearly demonstrable with EN4, an established marker of vascular endothelium.[18] Furthermore, in all disorders studied including those where ELAM-1 expression was widespread, staining of serial sections with EN4 demonstrated that only a minority of vessels were ELAM-1 positive.

The *in vivo* effects of recombinant human interferon-γ (rHu-IFN-γ) administered intradermally to normal human skin on ELAM-1 expression was studied. Despite the presence of a prominent perivascular lymphohistiocytic infiltrate,[19] and in contrast to strong induction of ICAM-1 (Fig. 6a) on both endothelium and keratinocytes, ELAM-1 was only weakly induced on vessels surrounded by inflammatory cells (Fig. 6b).

Double-labelling experiments were performed to correlate the presence of neutrophils with ELAM-1-positive endothelium. Neutrophils were identified with antibody to neutrophil elastase (NP57).[20] In psoriasis, neutrophils were (i) concentrated around ELAM-1 positive endothelial cells (Fig. 7a), (ii) adherent to the luminal surface of ELAM-1 positive cells (Fig. 7b) and (iii) noted between ELAM-1 positive cells, suggesting diapedesis. Interestingly, many ELAM-1 positive vessels were observed where neutrophils could not be demonstrated and in allergic contact and atopic dermatitis few neutrophils were observed in any sections despite strong expression of ELAM-1.

Discussion

This study has demonstrated for the first time that ELAM-1 is widely expressed on the surface of dermal

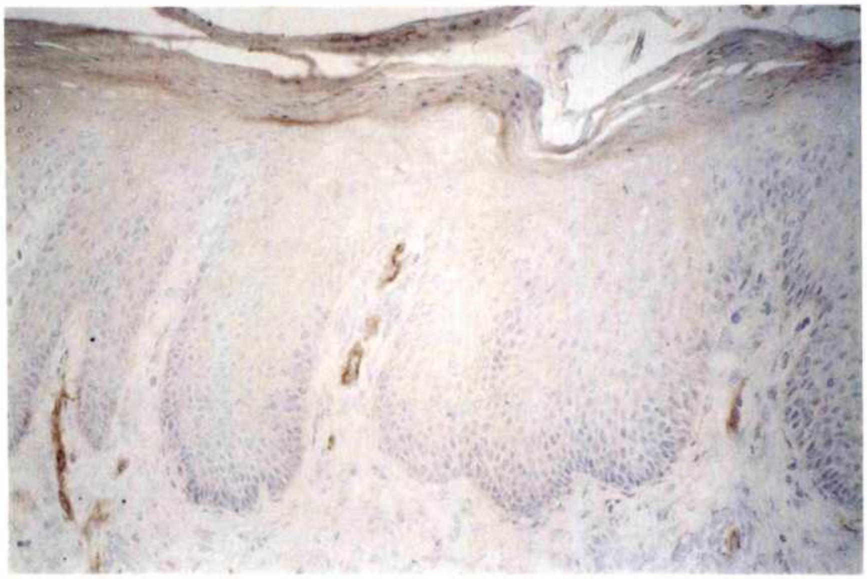

Figure 3. Psoriasis stained for ELAM-1: vessels in close proximity to lesional epidermis express ELAM-1 both in the presence and absence of adjacent inflammatory cells.

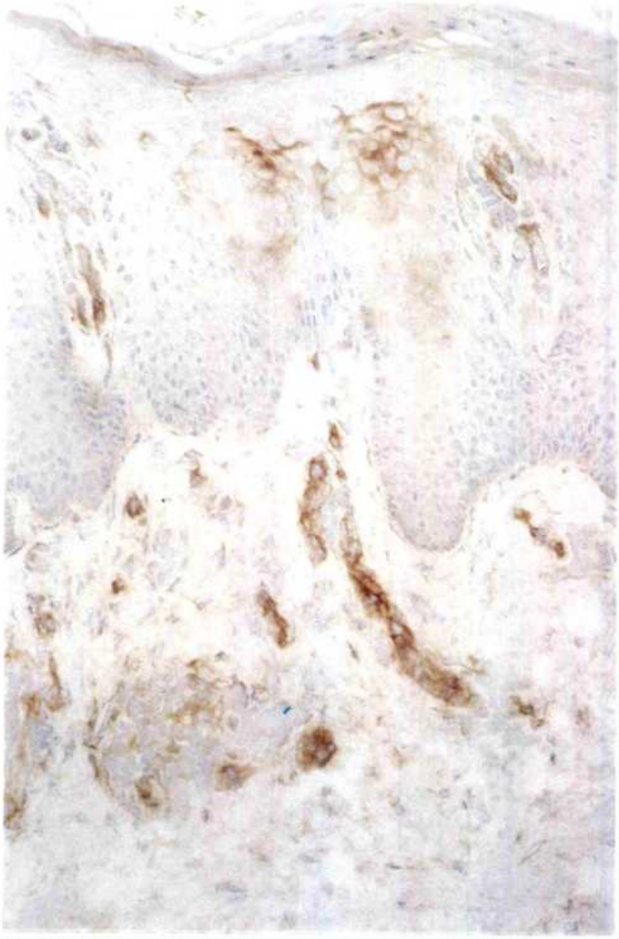

Figure 4. Psoriasis stained for ICAM-1: intense labelling of both dermal vascular endothelium and keratinocytes is observed.

vascular endothelial cells in cutaneous inflammatory and neoplastic diseases thus indicating that vascular endothelium is activated in these conditions. Our finding that endothelial ELAM-1 is sustained in states of chronic inflammation such as psoriasis is surprising, since *in vitro* ELAM-1 expression is transient even in the presence of continued cytokine stimulation.[8,21] This potential paradox may be resolved in one of two ways: firstly it is possible that the refractory period during which the endothelial cell cannot be stimulated to produce ELAM-1 is of finite duration after which they can once again express ELAM-1. Our observation that only a subset of endothelial cells express ELAM-1 when compared to EN4 expression is consistent with this hypothesis. Secondly, other as yet unidentified cytokines or combinations of cytokines may be able to stimulate continuous induction of the ELAM-1 gene.

In the disorders examined in the present study, ELAM-1-expressing vessels occurred particularly in areas of marked mononuclear cell accumulation, many of which were lymphocytes. It is unlikely that clustering of these cells occurred around these endothelial cells as a result of ELAM-1-mediated adhesive interactions as ELAM-1 is not an adhesive ligand for lymphocytes. Rather, it is probable that cytokines produced by perivascular mononuclear cells are responsible for ELAM-1 induction. Candidate cell populations for this role include macrophages, which may secrete both IL-1[22] and TNF-α[23] and lymphocytes which produce IFN-γ and lymphotoxin (TNF-β).[24] Keratinocytes have the ability to produce IL-1[25] and

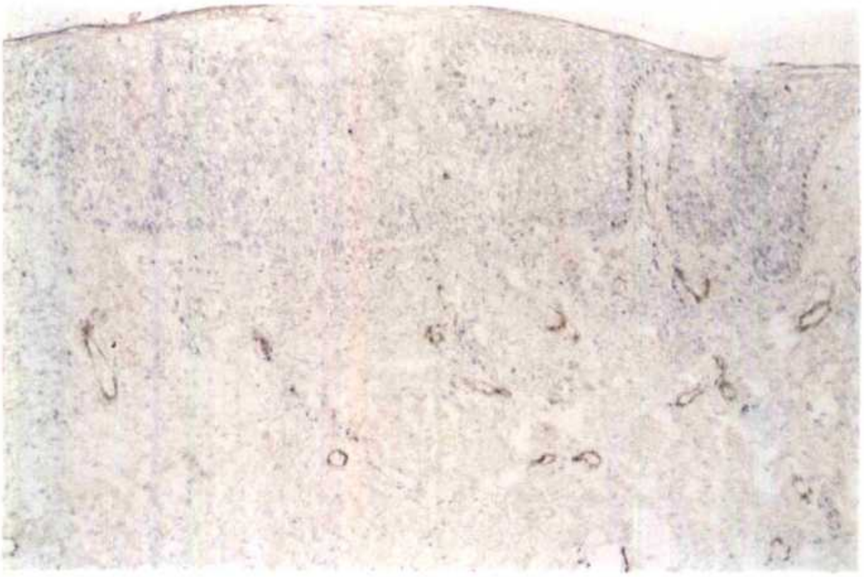

Figure 5. ELAM-1 is expressed on vessels within the infiltrate associated with Bowen's disease.

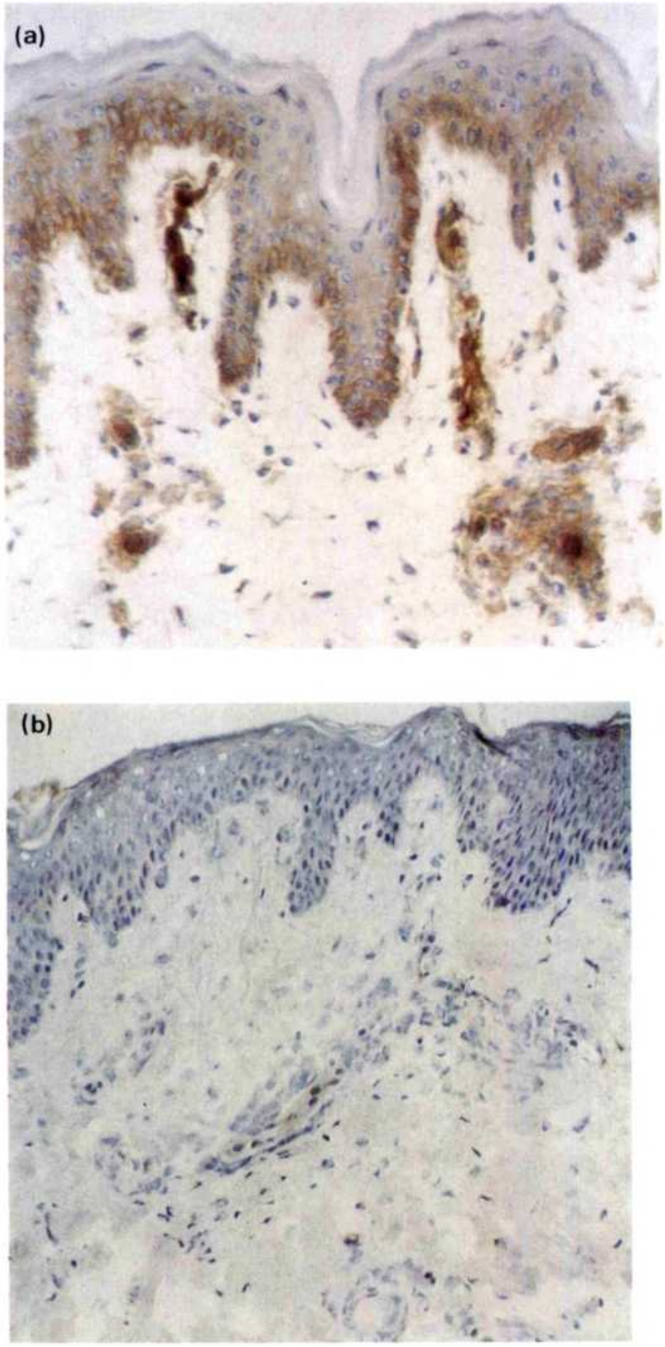

Figure 6. Normal skin following the injection of 30 μg recombinant human interferon gamma biopsied at day 6. (a) Strong induction of keratinocyte ICAM-1 expression, particularly in the basal layer and upregulation of dermal endothelium ICAM-1 expression is observed. (b) Under identical conditions, ELAM-1 is only weakly expressed on vascular endothelium, despite the presence of a marked infiltrate, and is not present on keratinocytes.

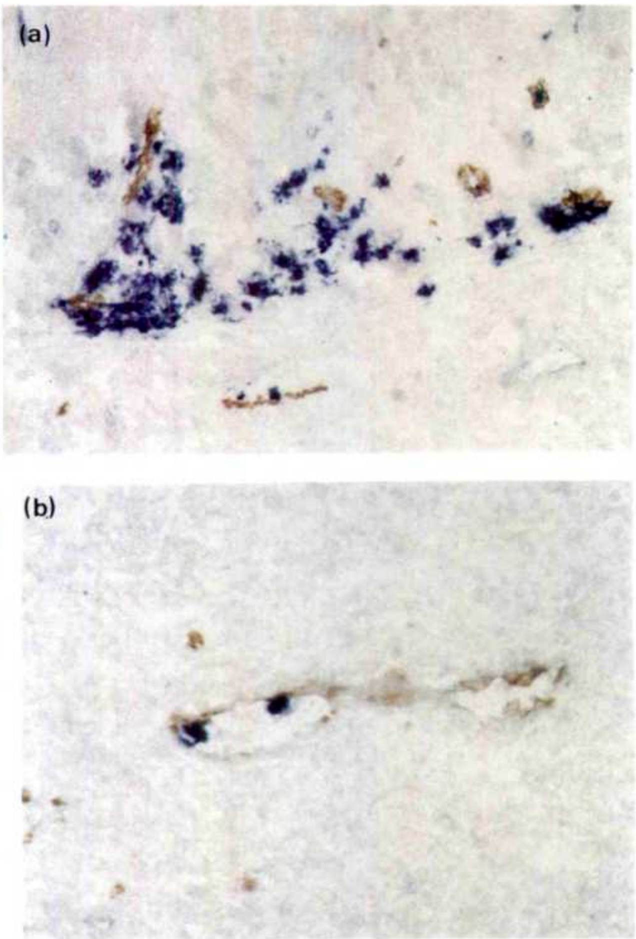

Figure 7. Psoriasis double-labelled with NP57 (blue stain) and 1.2B6 (brown stain). (a) Neutrophils are observed in close proximity to ELAM-1 positive vessels. (b) Neutrophils adherent to ELAM-1 positive dermal vessel.

TNF-α[26] both of which induce ELAM-1 and it is possible therefore that keratinocyte-derived cytokines have a role in inducing endothelial ELAM-1 expression. Our study provides some evidence for this: in psoriasis, in particular, ELAM-1 positive endothelial cells were observed in close proximity to lesional epidermis in the absence of inflammatory cells. ELAM-1 expression in these conditions provides some indirect evidence for functional interactions between perivascular mononuclear cells, resident cells, e.g. fibroblasts, dermal dendritic cells and keratinocytes, and the blood vessel wall.

Our finding of low levels of ELAM-1 expression in normal skin contrasts with previous findings.[7] Cross-reactivity of 1.2B6, although possible, is unlikely since it recognizes a single band corresponding to ELAM-1 on protein gel electrophoresis following immunoprecipitation.[14] A more likely explanation is the greater sensitivity of the assay used in the present study. *In-vivo* basal expression may occur either as a constitutive phenomenon, though this is not seen in cultured endothelial cells, or secondary to release of cytokines by adjacent cells including keratinocytes. An exaggeration of this phenomenon may be responsible for the spontaneous increase in endothelial ELAM-1 expression seen in the whole skin organ culture system in the absence of stimulatory cytokines.[11] Although we were unable to demonstrate a major effect of IFN-γ on ELAM-1 expression *in vivo*, the small increase in expression observed is probably a consequence of other cytokines locally induced by IFN-γ. In much the same way, ELAM-1 upregulation has been observed *in vivo* following IL-2 immunotherapy,[27] another cytokine without direct effect upon ELAM-1.

Despite our double-labelling studies demonstrating a link between neutrophil margination, diapedesis and ELAM-1 expression, other mediators of neutrophil adherence are likely to be of importance including the CD11/CD18 complex, ICAM-1 and GMP140.[28] Furthermore, recent evidence suggests that ELAM-1 mediates mononuclear phagocyte adhesion to endothelial cells. This adhesion to endothelial cells closely follows the time course of ELAM-1 induction[13] and the monocyte derived cell line U937 shows increased adherence to ELAM-1 transfected cells.[12] Thus, in addition to being an indicator of activated endothelium ELAM-1 may be of functional importance not only in the recruitment of neutrophils in the neutrophilic dermatoses but also in the recruitment of monocytes in those disorders where mononuclear cells predominate within the infiltrate, including atopic dermatitis and psoriasis.

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Inositol metabolism in mononuclear leucocytes from patients with atopic dermatitis

I.H.COULSON, G.R.HURT AND C.A.HOLDEN
Skin Laboratory, St Helier Hospital, Carshalton, Surrey SM5 1AA, U.K.

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Summary Elevated activity of cyclic adenosine monophosphate-specific phosphodiesterase (PDE) has been previously documented in the peripheral blood mononuclear leucocytes (MNLs) of patients with atopic dermatitis (AD). Because of the potential interactions of the cyclic nucleotide and inositol secondary messenger systems we sought to determine whether differences exist in inositol uptake and metabolism between atopic and normal MNLs. We found no difference in the uptake of tritiated inositol by MNL between 19 atopic patients and 16 non-atopic control subjects. However, after stimulation of MNLs by concanavalin A, there was a significantly greater metabolism of inositol to inositol-1-phosphate (IP_1) in the controls compared to the atopic MNLs; the mean percentage rise in the IP_1 fraction over baseline levels was 40% in the atopics, but almost 200% in controls after 2 h. Diminished inositol metabolism in atopic MNLs may explain the reduced cell-mediated immunity that characterizes the atopic state.

The cause of atopic dermatitis (AD), one of the commonest inflammatory dermatoses, is unknown. However, abnormalities of cyclic nucleotide metabolism have been demonstrated in peripheral blood mononuclear cells (MNLs), particularly monocytes, from patients with AD. Stimulation of MNLs with histamine, β adrenoreceptor agonists and E series prostaglandins results in an attenuated production of intracellular cyclic adenosine monophosphate (cAMP).[1] This appears to be the consequence of elevated activity of the enzyme cAMP-specific phosphodiesterase (PDE)[2] which catabolizes the cAMP to 5′ AMP, rather than a diminished activity of the enzyme adenylate cyclase.[3] The finding of this biochemical abnormality in the cord blood of infants born to atopic parents,[4] and an identical abnormality being found in the MNLs of a Basenji greyhound model of asthma and atopic dermatitis[5] has led to the suggestion that elevated PDE activity may be fundamental to the development of atopy.

However, *in vitro* stimulation of normal non-atopic MNLs with histamine has been shown to elevate PDE activity to a level similar to that seen in atopic MNLs,[6] suggesting that elevated PDE activity may be a secondary phenomenon. It is well documented that there are elevated levels of histamine in the skin of patients with AD,[7] and histamine release from basophils is increased when stimulated by anti-IgE.[8]

An intracellular secondary messenger system, the inositol cycle, has been identified which interacts with many of the other intracellular regulatory pathways (Fig. 1). Metabolism of phosphatidylinositol diphosphate (PIP_2) results in the liberation of diacylglycerol which can then activate protein kinase C.[9] In addition it has been shown that elevation of the activity of PDE can occur by phosphorylation of the enzyme by protein kinase C.[10] In some tissues (longitudinal smooth muscle of guinea-pig ileum) inositol uptake has been shown to be increased by histamine,[11] so that there are possible pathways whereby the inositol cycle may interact with and activate PDE.

The aim of this study was to determine whether the metabolism of inositol lipids in MNLs differ between normal subjects and those with atopic dermatitis. We also sought to determine the correlation between MNL PDE activity and inositol uptake by MNLs, and also to determine whether differences exist between the inositol metabolism of MNLs in normal and atopic subjects stimulated by histamine and the lymphocyte mitogen concanavalin A (Con A).

Methods

Before commencing this study, hospital ethical committee approval was granted and all subjects gave informed consent.

Correspondence: Dr I.H.Coulson.

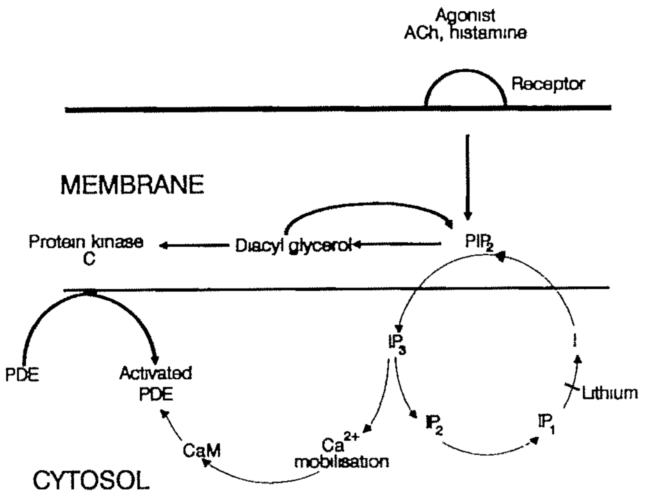

Figure 1. A simplified secondary messenger pathway showing the potential route whereby the inositol metabolites may interact with cAMP phosphodiesterase. PIP_2, phosphatidylinositol diphosphate; IP_3, inositol triphosphate; IP_2, inositol diphosphate; IP_1, inositol monophosphate; I, inositol; PDE, cAMP specific phosphodiesterase; CaM, calmodulin; ACh, acetyl choline.

Atopic dermatitis sufferers

Nineteen young adults with AD fulfilling the criteria of Hanifin and Rajka[12] were studied. All avoided topical steroid therapy and antihistamines for 1 week and beverages containing methyl xanthine (known to inhibit PDE activity) for 12 h prior to venesection. No patients were receiving oral corticosteroids.

Non-atopic control subjects

Sixteen young adults with no personal or family history of atopy (history of asthma, eczema or seasonal rhinitis) were studied. All were prick-test negative to a series of nine common reaginic antigens and none were taking concurrent medication. All avoided beverages containing methyl xanthine for 12 h prior to venesection.

MNL separation

In all cases MNL separation was undertaken within 10 min of venepuncture. Blood (20 ml) mixed with 100 units of sodium heparin was diluted with an equal volume of phosphate-buffered saline (PBS) and MNLs were separated by Hypaque–Ficoll centrifugation. The cells were washed three times in PBS containing 0·3 mM ethylenediamine tetra-acetic acid (PBS-EDTA), and centrifuged at 350, 300 and 250 *g* in a swing-out head to remove platelets. After counting using a haemocytometer and methylene blue dye to ensure there was no platelet or granulocyte contamination, the cells were suspended at 5×10^6 cells/ml in Gey's balanced salt solution (GBSS, Gibco) with 5% fetal calf serum. The MNLs, comprising 80% lymphocytes and 20% monocytes and less than 1% granulocyte contamination were either used fresh for inositol metabolism experiments, or diluted in GBSS, and frozen at −70°C until required for the PDE assay.

Tritiated inositol uptake by fresh MNLs

Neat [^{3}H]-inositol (10 μl) (specific activity 66–67 Ci/mmol, Amersham International, Amersham) was pipetted onto a 1 ml Dowex formate column and washed through with 1 ml of deionized water. Fifty microlitres (0·5 Ci) of the inositol eluate was mixed with 2×10^5 MNL cells suspended in 200 μl of GBSS with 5% fetal calf serum and 50 μl of 60 mM lithium chloride in GBSS. Lithium was used to prevent the conversion of inositol monophosphate (IP_1) to inositol and cause accumulation of IP_1. Preliminary experiments had determined 10 mM to be the optimal lithium concentration.

The mixture was incubated at 37°C for 24 h in a humidified incubator with a 5% CO_2/air mixture. Subsequently the cells were washed three times in PBS, and resuspended in 0·5 ml of distilled water. The cells in suspension were disrupted by freezing and thawing three times, liquid scintillation fluid was added, and the radioactivity counted using a Rackbeta liquid scintillation counter.

Concanavalin A stimulation

[^{3}H]-inositol (50 μl) (prepared as described above) was mixed with 2×10^5 cells in 200 μl of GBSS with 5% fetal calf serum and incubated in a humidified incubator at

Table 1. Buffers used in separation of inositol metabolites

Buffer		Principal metabolite eluted
1	5 mM myoinositol	Inositol (I)
2	60 mM sodium formate and 5 mM disodium tetraborate	Glycerophospho-inositol (GPI)
3	0·1 M formic acid and 0·2 M ammonium formate	Inositol-1-phosphate (IP_1)
4	0·1 M formic acid and 0·4 M ammonium formate	Inositol 1,4-diphosphate (IP_2)
5	0·1 M formic acid and 1 M ammonium formate	Inositol 1,4,5-triphosphate (IP_3)

37°C with 5% CO_2/air for 24 h. The cells were then washed three times with 2 ml of PBS, resuspended in 200 μl of GBSS with 5% fetal calf serum mixed with 50 μl of 60 mM lithium chloride and incubated for a further 30 min. Fifty microlitres of a 300 μg/ml solution of concanavalin A in GBSS or plain GBSS was added and the reaction stopped at 0, 30, 60 or 120 min by the addition of 200 μl of trichloroacetic acid. Cells were disrupted by freeze thawing three times and inositol metabolites separated by ion exchange chromatography.

Separation of inositol metabolites

After 24-h incubation of MNLs with [^{3}H]-inositol, lithium and PBS, the inositol metabolites were separated by Dowex formate ion exchange column chromatography. Five different buffers were used (Table 1). Each eluate (0·5 ml) was added to 3 ml of scintillant and the level of radioactivity measured.

cAMP phosphodiesterase assay

Thawed 2-ml aliquots of MNLs were sonicated at 20 kHz for 25 s at 4°C using an Ultrasonic sonicator and diluted to 1×10^6 cells/ml with GBSS and assayed for PDE activity using a modification of the procedure of Thompson *et al.*[13] The diluted homogenate (200 μl) was then added to 200 μl of cAMP substrate (0·05 μM) with 20,000 c.p.m. of [^{3}H]-cAMP (Amersham International, Amersham) in 40 mM Tris HCl buffer (pH 8·0) containing 3·75 mM β-mercaptoethanol and 50 mM $MgCl_2$. Previous kinetic studies[3,14] in adults have shown a greater discrimination between atopic and non-atopic individuals' MNL PDE activities at low substrate concentrations; a cAMP concentration of 0·05 μM gave the optimal distinction between these two groups. Blank samples contained 200 μl of GBSS and 200 μl of cAMP substrate. The samples were incubated at 30°C for 10 min and then transferred to a boiling waterbath for 90 s, cooled and frozen.

Having returned the reaction mixtures to 4°C, 100 μl of 5′ nucleotidase (0·45 units/ml in 40 mM Tris HCl buffer, pH 8·0) was added and then incubated at 30°C for 10 min. The [^{3}H]-adenosine was separated from the reaction mixture using 0·7 ml Dowex 1 × 2–400 Cl^- anion-exchange column (applied as 1·4 ml of a 50% v/v slurry in distilled water). The radioactive adenosine was eluted with 1 ml + 2 × 2·5 ml of distilled water. The eluate (0·5 ml) was counted in 3 ml of scintillation fluid using a LKB1212 Rackbeta liquid scintillation counter.

Having determined the protein present in each sample using the method of Lowry *et al.*,[15] the PDE activity was expressed as pmol cAMP hydrolised/min/mg protein.

Statistics

Results are expressed as the mean ± SEM. Student's *t*-test was used to determine statistical significance.

Results

MNL inositol uptake

Although the mean inositol uptake by atopic MNLs (4484 ± 2778 c.p.m.) was greater than that by the control cells (4019 ± 2884) this did not reach statistical significance ($P > 0{\cdot}2$).

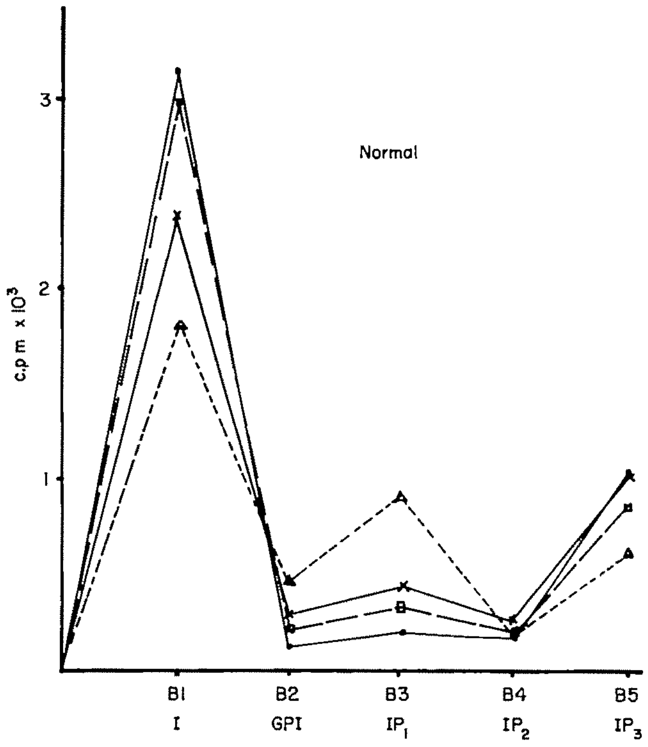

Figure 2. Illustration of the effect of stimulation of a normal individual's MNLs on inositol and its metabolites with Con A. B1–B5 represents the sequence of buffers applied eluting inositol (I), glycerophosphinositol (GPI), inositol phosphate (IP_1), inositol diphosphate (IP_2) and inositol triphosphate (IP_3), respectively. Commensurate with a progressive fall in the activity in B1 eluate corresponding to inositol (I), there is a progressive rise in the activity in B2 eluate corresponding to inositol monophosphate (IP_1). ●——●, 0 min; □– – –□, 30 min; ×——×, 60 min; △– –△, 120 min.

Correlation of MNL PDE activity and [^{3}H]-inositol uptake

We found a statistically significant elevation of atopic MNL PDE activity ($9{\cdot}11 \pm 2{\cdot}38$ pmol/min/mg protein) compared to non-atopic individuals MNL PDE ($6{\cdot}9 \pm 1{\cdot}45$ pmol/min/mg protein, $P<0{\cdot}05$). In both the atopic and control MNLs there was no correlation between the inositol uptake and the PDE activity.

Effects of concanavalin A stimulation of MNLs on inositol metabolites

Stimulation of normal MNLs with Con A produced a progressive increase in the radioactivity in both the second, but particularly the third buffer eluate, corresponding to a progressive conversion of tritiated inositol to IP_1. Data from a representative control individual is depicted in Figure 2. Commensurate with this there was a decrease in the inositol pool with time.

In contrast, in the atopic MNLs an elevation of the counts in the second and third eluates was not seen after Con A stimulation. Data from a representative atopic patient is depicted in Figure 3. However, between individual variability was great, so that results are expressed as the percentage rise in counts per minute over the baseline unstimulated count. The mean percentage rise in the IP_1 fraction over the baseline (time 0) was 40% after 2 h in the atopics ($n=8$) and this did not reach statistical significance, but was almost 200% in the MNLs from controls ($n=7$), $P<0{\cdot}005$ (Fig. 4). The increase at 2 h in the control group was significantly greater than that of the atopic group ($P<0{\cdot}025$).

Histamine stimulation

Using the same methodology as above, we were unable to demonstrate any increase in inositol turnover following histamine stimulation at concentrations of 10^{6}–10^{-3} M in MNLs of five normal individuals.

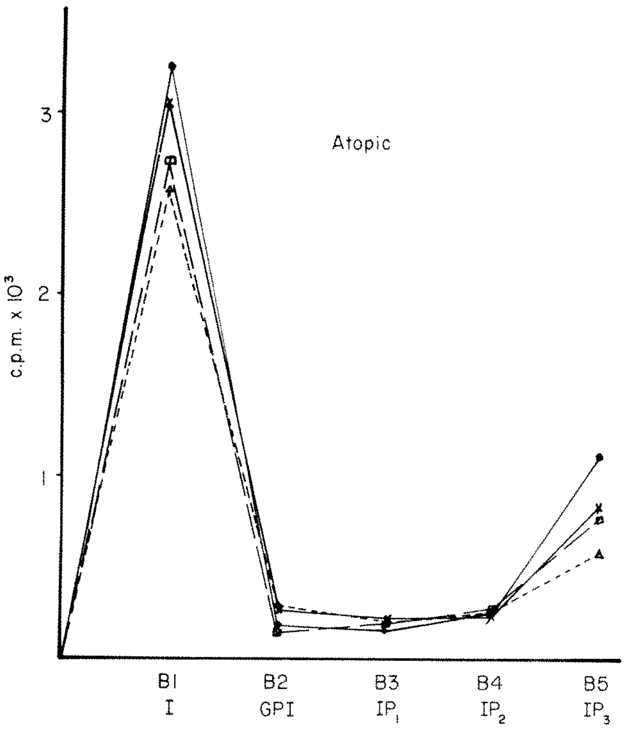

Figure 3. Illustration of the effect of stimulation of a typical atopic patient's MNLs with Con A, demonstrating no fall in inositol values and no accumulation of inositol monophosphate. B1–B5 represents the sequence of buffers applied eluting inositol (I), glycerophosphinositol (GPI), inositol phosphate (IP_1), inositol diphosphate (IP_2) and inositol triphosphate (IP_3), respectively. ●——●, 0 min; □- - -□, 30 min; ×——×, 60 min; △- - -△, 120 min.

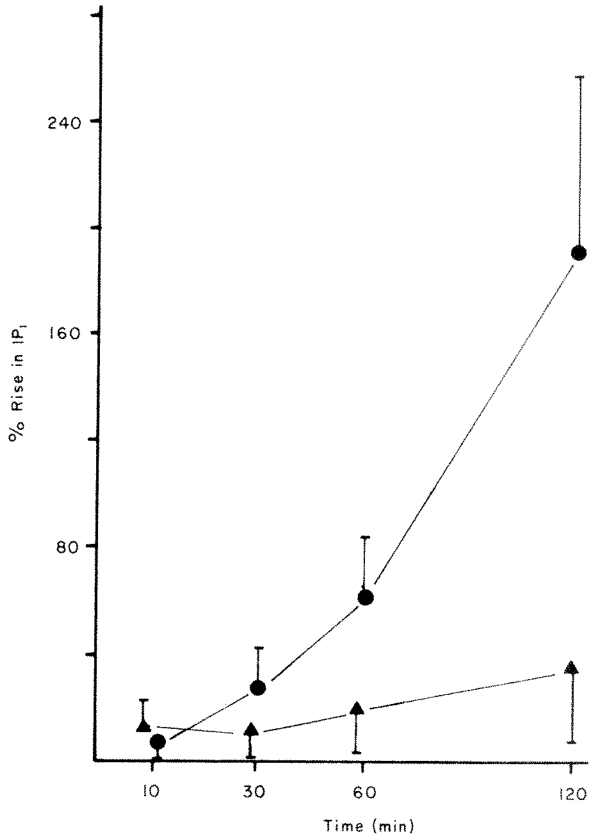

Figure 4. Comparison of the percentage rise in activity of the third buffer eluate (corresponding to IP_1) after stimulation of normal and atopic MNLs with Con A (mean ± SEM). In the normal MNLs there is a mean rise of 200%, in the atopic MNLs the rise is significantly smaller ($P<0{\cdot}025$). ●, normal ($n=7$); ▲, atopic ($n=8$).

Discussion

On initiating this study, we expected to find an elevation of the inositol turnover that would explain the elevation in the PDE that we and others have documented in the MNLs of patients with atopic dermatitis.[2,14] It was anticipated that exposure to histamine may stimulate inositol turnover resulting in the release of diacylglycerol which activates protein kinase C, which in turn may phosphorylate and activate PDE. However, our results show that the uptake of inositol by atopic and non-atopic control MNLs does not differ. In addition, we found no correlation between PDE activity and inositol uptake in either atopic or control MNLs.

Galey *et al.*[16] similarly showed no difference in total inositol uptake between atopic and control MNLs, but they were able to show a significantly greater unstimulated incorporation of inositol into inositol phosphates and phosphinositides in the atopic MNLs. Our unstimulated incorporation of inositol into inositol phosphates was not significantly raised. Also, attempts to demonstrate stimulation of inositol turnover by histamine in normal MNLs were unsuccessful.

However, stimulation of inositol metabolism by Con A demonstrated significant differences between control and atopic MNLs. The results demonstrate reduced incorporation of $[^{3}\mathrm{H}]$-inositol into inositol phosphates after Con A stimulation in the atopics compared to controls. This finding is of considerable interest. Diminished atopic peripheral blood lymphocyte transformation on mitogen stimulation has been recognized for some years[17,18] and this correlates well with the reduced cell-mediated immunity seen in atopic dermatitis manifested as an increased susceptibility to viral and fungal infections coupled with reduced susceptibility to the initiation of allergic contact sensitisation.[19] The finding of reduced Con A-stimulated inositol incorporation in atopic MNLs may be an explanation for these findings on a biochemical level. One other possibility is that Con A may have differential effects on different subsets of the mononuclear cell population. Most evidence suggests that Con A acts mostly on suppressor T lymphocytes,[20] which are known to be present in diminished numbers in AD. This may account for the differences in inositol metabolism seen between the two groups.

It would appear that multiple abnormalities of secondary messenger systems are present in atopic MNLs. Cyclic AMP hyporesponsiveness secondary to elevated PDE activity is clearly documented, and has been proposed as an explanation for the reaginic hyperreactivity seen in atopy. PDE inhibition using the agent RO 20-1724 reduces the hypersynthesis of IgE by atopic dermatitis cells *in vitro*[21] and histamine release by atopic leucocytes is strongly correlated to their PDE activity.[22] Our finding of reduced Con A-stimulated inositol responses may explain the reduced suppressor T-cell function seen in AD[23] and it could contribute further to increased IgE production. Diminished inositol responses to other mitogens may also be present in AD and this requires further investigation.

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Melanocytic lesions and melanocyte populations in human epidermis

MARY E.FALLOWFIELD, REGINA K.CURLEY* AND M.G.COOK

*Departments of Histopathology and *Dermatology, St George's Hospital, Blackshaw Road, London SW17 0QT, U.K.*

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Summary Basal melanocytes were counted and atypia assessed on an arbitrary scale in punch biopsies from the sun-exposed extensor aspect of the forearm of normal skin and from the covered skin of the buttock of patients with pigmented naevi and control subjects. The difference in melanocyte counts and in the presence of atypia between sun-exposed and covered skin was statistically highly significant. The only other difference was between melanocyte counts in covered skin from those with multiple atypical naevi and all other groups; the counts in the former were greater than those in the latter. No further difference was attributable to sun exposure, skin type or diagnostic group. Some degree of melanocyte atypia was seen in approximately half of the biopsies of sun-exposed skin, but atypia was seen in only six of 84 biopsies of covered skin. In each case atypia was present in the corresponding forearm biopsy and all six subjects had pigmented lesions (four with melanoma and two with multiple atypical naevi). It seems possible that while increased melanocyte counts in covered skin could correlate with the presence of atypical naevi, atypia of covered epidermal melanocytes could possibly relate to melanoma risk.

The melanocyte distribution in normal human epidermis and the responses of these melanocytes to ultraviolet irradiation have been studied by several groups with broadly comparable results.[1–5] In adult Caucasians a greater density of melanocytes is seen in the epidermis of the face and forearms than in other parts of the body, all of which have similar numbers of melanocytes. This distribution of melanocytes is thought to reflect the greater sun exposure of the skin of face and arms.[3,4,6] The effects of short-term ultraviolet-light (UV) exposure[1,7] and chronic UV[5,6,8] and sun exposure[2,3] have been studied both in experimental animals and in human subjects. It has been shown that chronic sun exposure induces melanocyte proliferation,[9] sometimes leading to the development of solar lentigines, which have many histological features in common with melanocytic dysplasia[10] and atypical forms of melanocytes are also seen.[2] Greater degrees of sun exposure may induce melanocyte destruction with loss of pigment cells from the epidermis.[2]

As ultraviolet irradiation induces melanocyte proliferation and atypia, it has been implicated in the development of melanoma. In mice and, more recently, in a group of human volunteers, Rosdahl and his co-workers have demonstrated that melanocyte counts in shielded skin also increase after UVB exposure and have suggested that this response may be involved in the aetiology of melanoma of non-sun-exposed skin.[5,8]

Increased numbers of basal melanocytes have been observed at the resection margins of wide excisions of melanoma up to 5 cm from the edge of the tumour.[11] Some increase in melanocyte density has also been reported close to benign and atypical (dysplastic) naevi and this has led to the concept of an 'expanded and activated melanocyte system' in patients with melanoma or multiple atypical naevi.[12,13]

We have studied a series of patients attending the Pigmented Lesion Clinic (PLC) at St George's Hospital, in order to ascertain whether they also exhibited elevated melanocyte counts at sites distant from their pigmented lesions; how the density of their melanocytes was affected by sun exposure and whether skin type, presence of naevi, or sun exposure were associated with cytological atypia.

Methods

Patient selection

Volunteers were recruited from patients attending the PLC, between January 1987 and December 1988, who had a histologically proven diagnosis of melanoma or

Correspondence: Dr M.E.Fallowfield, Department of Histopathology, St George's Medical School, Cranmer Terrace, London SW17 0RE, U.K.

naevi with melanocytic dysplasia, with or without melanoma. A control population was recruited from randomly selected patients attending the general dermatology clinics, who were without atypical pigmented naevi or inflammatory skin disorders. The control group was not formally matched with respect to age, sex or skin type, but had a broadly similar distribution of these parameters to that seen in the study population.

Skin type was assessed by one of the authors (R.K.C.) using established criteria of the Fitzpatrick classification,[14] according to the colour of unexposed skin and tanning and burning responses to sun exposure. Hair colour and eye colour were also recorded.

Sun exposure was assessed by the same observer (R.K.C.), following direct questioning regarding time spent sunbathing, engaging in outdoor pursuits and residence in tropical climes, and scored from 1 (below average—never holidays abroad nor sunbathes in this country) to 4 (excessive—residence in the tropics for 6 months or more).[13]

The melanocyte counts were normally distributed, so differences between the various groups could be sought by means of analysis of variance. The paired data for exposed and covered melanocyte counts were compared using Student's paired *t*-test and differences in frequency of melanocyte atypia between the various groups were sought using contingency tables and the chi-squared test.

Biopsy and melanocyte counts

For each subject a 3-mm punch biopsy was taken from the proximal part of the extensor aspect of the right forearm; a second biopsy was obtained from an area of double-covered, non-sun-exposed skin from the right buttock. Biopsies were fixed in 10% phosphate-buffered formalin in coded pots, processed and embedded in paraffin wax. Sections (2 μm) were cut and stained with haematoxylin and eosin. Biopsies were assessed without breaking the code and without knowledge of the sex, age, skin type or diagnostic category. The number of basal melanocytes associated with 200 basal keratinocytes was counted in each biopsy. During the study, sections of 40 randomly chosen biopsies were stained with S100 to demonstrate melanocytes. A good correlation was obtained between melanocyte counts from haematoxylin and eosin-stained sections and those from S100-stained sections, so this additional technique was not performed on every biopsy. Atypia was assessed on the basis of enlarged angular melanocyte nuclei seen in haematoxylin and eosin sections and graded on a 0, +, + + scale; although this was an arbitrary technique it appeared to be reproducible, within the limits of this study. The estimate of the grade of atypia was similar on at least three occasions when the biopsies were reassessed 'blind'. Both pathologists assessed the melanocytes in each biopsy separately and subsequently compared their findings; there was a high degree of concordance between the assessments.

Results

Biopsies were taken from 84 subjects, 31 males (mean age 36·8 years) and 53 females (mean age 38·2 years). Melanocyte counts ranged from 27 to 55 per 200 keratinocytes for sun-exposed forearm skin, with one very low count of 6 from an individual with blond hair, blue eyes, type 2 skin and a personal and family history of melanoma. Whilst no melanocyte atypia was seen in this biopsy, keratinocyte atypia and solar elastosis were seen. The highest melanocyte count was seen in the exposed skin of a subject with hazel eyes, fair hair, type 2 skin and a personal history of melanoma. For covered buttock skin, melanocyte counts ranged from 10 to 35.

The melanocyte counts were normally distributed and were analysed (Table 1) according to diagnostic group (1, melanoma; 2, melanoma with multiple naevi showing the histological features of melanocytic dysplasia; 3,

Table 1. Melanocyte counts according to skin type, diagnostic group and sun-exposure score for sun-exposed and covered skin (mean ± standard deviation)

	Number	Covered skin	Exposed skin
Skin type			
1	11	21·1±4·1	34·2±5·6
2	38	21 4±6·5	34·9±9·0
3	33	23·0±6·9	39·1±9·3
4	2	20·0±7·1	35·5±3·53
		P=0 6686	*P*=0·1782
Diagnostic group			
1	20	20 9±6 7	34·6±12 0
2	7	18·6±2·6	36·6±7·1
3	36	24·7±5 9	37·6±8 4
4	21	19·4±6·1	35·6±6 3
		P=0·0042	*P*=0·5606
Sun-exposure score			
1	5	19·8±6·0	33·4±7·3
2	52	21 0±5·4	35·3±7·7
3	11	23·6±7·7	42·0±12·9
4	16	24·7±7·8	37·4±8·6
		P=0·1416	*P*=0·1091

multiple naevi with features of melanocytic dysplasia without personal or family history of melanoma; and 4, control); skin type (1–4; no subjects with skin type 5 or 6 were included in this study) and sun exposure, by analysis of variance. The paired data for exposed and covered melanocyte counts were compared by Student's *t*-test. There was no significant difference between counts for exposed or covered skin with regard to skin type or sun exposure score. There was no difference in melanocyte counts for exposed skin between the diagnostic groups, but there was a significant difference ($P<0{\cdot}0001$) between covered skin melanocyte counts for those with multiple atypical naevi (Group 3) and the other diagnostic groups. The melanocyte counts in Group 3 were higher than expected. This difference was not accounted for by skin type nor by sun exposure and appeared therefore to be a characteristic of the epidermal melanocytes of those with multiple atypical naevi (Table 1). As expected there was a highly significant difference between the melanocyte counts from exposed and covered skin ($P<0{\cdot}0001$).

Forty-three of 168 biopsies were thought to show some degree of melanocyte atypia; 37 of 84 exposed skin biopsies and six of 84 covered biopsies. The greatest melanocyte atypia was seen in sun-exposed skin from a control subject, a 75-year-old woman with brown eyes and fair hair and in sun-exposed skin from a 26-year-old female with green eyes, auburn hair and previous melanoma. Melanocyte atypia in covered skin was always seen in conjunction with atypia of exposed skin and was only seen in subjects with pigmented skin lesions: one with a history of melanoma and multiple atypical naevi; three with previous melanoma, but no naevi and two with multiple atypical naevi. The χ^2-test was used to analyse the results of the atypia assessments and as with the counts, there was no difference between the frequency of melanocyte atypia with skin type, sun exposure or diagnostic category, for covered or exposed skin biopsies, but there was a highly significant difference between covered and exposed biopsies ($P<0{\cdot}0001$).

Discussion

In this study we found that there was, on average, one basal melanocyte for every 10 basal keratinocytes in double-covered buttock skin. This result is similar to that obtained by other workers.[12,15,16] Sun-exposed epidermis possessed approximately twice as many basal melanocytes as covered skin, a statistically significant difference, in accord with the work of Gilchrest and colleagues,[3] who demonstrated twice as many epidermal melanocytes in skin from the outer aspect of the arm as in skin from the inner aspect. Furthermore, they suggested that melanocyte density is proportional to cumulative sun exposure. In our study, melanocyte counts appeared to peak at approximately one to every four keratinocytes, irrespective of the sun-exposure score. It is likely that forearm skin receives much unnoticed sun exposure even in those who avoid sunbathing and this may be sufficient to induce maximal melanocyte proliferation in many individuals.

It has recently been reported that an increase occurs in melanocyte numbers in covered epidermis some days after UV exposure of surrounding skin.[5] We found no difference in melanocyte counts with sun-exposure score, for covered or exposed skin, which may in part be due to the difficulty in assessing the degree of sun exposure retrospectively. In addition, for many of our subjects some months or longer had elapsed between their last episode of sun exposure and the time of biopsy, which may have allowed the melanocytes in the covered skin to regain their basal levels. We could find no difference in basal melanocyte counts between skin types, in agreement with previously published studies,[17,18] for either covered or sun-exposed skin.

While there was no difference in sun-exposed epidermal melanocyte counts between diagnostic groups, Group 3 (multiple atypical naevi without melanoma) exhibited significantly higher melanocyte counts in covered skin than those seen in any other diagnostic category. This is an interesting finding and could perhaps indicate increased melanocyte proliferation in those with atypical naevi but without melanoma. Such a phenomenon could be a manifestation of the 'expanded and activated melanocyte system' proposed by MacKie.[13]

As with melanocyte counts, there was no difference in melanocyte atypia with sun exposure score, skin type or diagnostic group; the only significant difference was between exposed and covered skin. Mitchell[2] has described atypical forms of melanocytes in chronically sun-exposed skin, similar to those observed in this study. Wong[11] found occasional atypical melanocytes in sun-exposed skin adjacent to melanomas, but ascribed these to a field change in melanocytes surrounding melanomas, rather than to sun exposure. We had expected to see more melanocyte atypia in those with higher sun-exposure scores and in patients with melanoma or atypical naevi, those who could be expected to have expanded and activated melanocyte systems,[13] compared with controls, but no evidence of this could be found.

Some degree of melanocyte atypia was seen in almost half of the control subjects' forearm biopsies. As with melanocyte counts, there was no difference in the amount of atypia seen in sun-exposed melanocytes with sun-exposure score, skin type or diagnosis. Atypia of covered epidermal melanocytes was present in the biopsies of only six of 84 subjects, three of whom had sporadic melanomas; one had a melanoma with multiple atypical naevi and two exhibited multiple atypical naevi. Although this group was very small, the presence of covered atypia appears to correlate with the presence of melanoma and atypical naevi. This finding could possibly indicate patients with abnormal melanocytes who may have an increased risk of developing melanoma and could be further investigated by means of a prospective study.

The changes described as atypia in this study referred to an increase in nuclear size and angularity in haematoxylin and eosin-stained sections. It was not possible to obtain detailed measurements of the nucleus and perikaryon of melanocytes, as would have been possible in Dopa-haematoxylin sections,[15] but unfortunately logistic problems precluded use of this technique. The degree of melanocyte atypia grade '+' was frequently seen in sun-exposed skin (50% of forearm biopsies) and is unlikely to be of any significance, but melanocytes with '++' atypia showed an obvious increase in nuclear size and cytoplasm compared with normal melanocytes. Apart from the significant difference between exposed and shielded skin, the other statistically significant result was seen in covered buttock skin, where increased melanocyte numbers were present in those with multiple atypical naevi, compared to those with melanoma and control subjects. In addition, covered melanocytes only exhibited any degree of atypia in those with pigmented lesions. This raises the possibility that multiple atypical naevi differ in aetiology from melanoma or melanoma with multiple atypical naevi, as the former exhibit increased numbers of melanocytes without atypia, whereas the latter, a very small group in this study, exhibit atypia without increased numbers of melanocytes in covered skin. It has been proposed that clinical and histological features of melanocytic dysplasia are commonly, but not always present in naevi acquired during adult life.[19] Thus, subjects included in this study with multiple atypical naevi may have had adult-type acquired naevi with minimal risk for melanoma, reflecting some generalized increased proliferation of epidermal melanocytes. By contrast, some of those with melanoma exhibited mild atypia of their covered melanocytes and it is possible that their melanocytes could be abnormal throughout the skin. Of the six buttock biopsies exhibiting mild melanocyte atypia, four were from subjects with a history of melanoma and two from individuals with atypical naevi. It will be interesting to follow the latter patients to see whether they develop melanoma; if so, biopsy of non-sun-exposed skin could perhaps be of some value in deciding which patients with features of dysplastic naevi would benefit from clinical follow up.

Acknowledgments

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VLA protein expression on epidermal cells (keratinocytes, Langerhans cells, melanocytes): a light and electron microscopic immunohistochemical study

G.ZAMBRUNO, V.MANCA, M.L.SANTANTONIO, D.SOLIGO* AND A.GIANNETTI
*Department of Dermatology, University of Modena and * Medical Sciences Institute, University of Milan, Italy*

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Summary

The very late antigens, or VLA proteins, are a family of cell surface heterodimers ($\alpha_{1-6}\beta_1$) that mediate cell adhesion to specific components of the extracellular matrix (collagens, fibronectin, laminin). In normal human epidermis, the common VLA β_1 subunit is expressed on basal keratinocytes (BK), Langerhans cells (LC) and melanocytes. By means of light and electron microscopic immunostaining procedures, we have investigated the distribution of VLA $\alpha_{1,2,3,4,6}$ subunits on normal human adult and foetal epidermal cells. α_1 antigen expression was not observed on any epidermal cell type. Both during foetal development and in adult epidermis, α_2 and α_3 were strongly expressed on the cell membrane of BK, while α_6 was mainly expressed at their dermal pole. These different patterns of distribution suggest that the α_6 subunit may mediate BK anchorage to the basement membrane zone, while the α_2 and α_3 subunits may also be involved in intercellular adhesion. Moreover, with immunoelectron microscopy, LC were seen to be weakly α_4 and α_6 positive and melanocytes were α_3 and α_6 positive. Thus, VLA proteins are expressed by epidermal cells in a cell-type-specific pattern that could be related to particular functional roles of these proteins.

The very late antigens, or VLA proteins are a family of transmembrane heterodimeric glycoproteins, of which VLA-1 and -2 were first characterized as antigens appearing during *in-vitro* activation of T lymphocytes.[1] Subsequently, four additional VLA molecules (VLA-3–6) were identified.[2,3]

The VLA family together with two other groups of surface molecules, the Leu–CAM family[4] and the cytoadhesins,[5] constitute the integrin superfamily of cell adhesion molecules (I-SCAM).[6] The I-SCAM includes at least 11 receptors which are involved in cell–cell and cell–matrix interactions.[6] Each integrin receptor consists of two different subunits, called α and β. VLA proteins are composed of distinct α subunits (α_{1-6}) of 140–200 kDa, non-covalently associated with a common β_1 subunit of MW 110 kDa. Most VLA complexes represent receptors for the extracellular matrix components fibronectin, laminin and collagens, and they are variously expressed on nearly all cell types.[7] VLA-1 ($\alpha_1\beta_1$) is the only VLA protein for which a ligand has not yet been discovered. However, an integrin heterodimer of the same molecular weight as VLA-1 has been shown to function as a laminin receptor on a rat neuroblastoma cell line.[8] VLA-2 ($\alpha_2\beta_1$) has been identified as a collagen receptor on platelets and fibroblasts[7] and recently also as a laminin receptor on human umbilical vein endothelial cells, suggesting a cell-type specific functional heterogeneity of this integrin.[9] The VLA-3 ($\alpha_3\beta_1$) heterodimer appears to have multiple ligands, fibronectin, laminin and collagens,[7] and to be expressed on most epithelial and mesenchymal cells. VLA-4 ($\alpha_4\beta_1$) has recently been shown to represent a fibronectin receptor on T lymphocytes[10] and VLA-5 ($\alpha_5\beta_1$) is a recognized fibronectin receptor on a number of cell types, including leucocytes, platelets and fibroblasts.[7,10] The α_6 subunit represents the laminin receptor on platelets, where it is associated with the IIa (β_1) glycoprotein.[3] Moreover, the α_6 subunit has been found in a different heterodimeric complex, together with a newly described β_4 subunit.[11] The $\alpha_6\beta_4$ complex can be demonstrated on various epithelial cell types.[11] Thus, VLA proteins appear to be differentially expressed and to have distinct functional roles on various cell types.

In the skin, laminin and type IV collagen are major components of the basement membrane zone (BMZ), to which the epidermal basal cells are firmly attached.[12]

Correspondence: Dr. G. Zambruno, Clinica Dermatologica, Universitá di Modena, Via del Pozzo N. 71, 41100 Modena, Italy.

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Fibronectin plays an important role in wound healing by enhancing the migration of keratinocytes.[12] Extracellular matrix components are not only involved in keratinocyte adhesion and migration, but also in the regulation of growth and differentiation.[13]

The present study investigated the expression and distribution of the individual VLA subunits on normal human epidermal cells from adult and foetal skin, using immunohistochemical techniques with both light and electron microscopy.

Methods

Skin specimens

Punch biopsies were obtained from clinically normal skin on the buttocks and forearms of eight healthy volunteers, with an age range of 24–69 years. Skin slices were obtained from surgical specimens obtained from the breast and abdomen using a keratotome. Human foetal skin was obtained from the limbs and trunk of 10 embryos of 10–21 weeks gestational age (GA). The biopsies of both adult and foetal skin were immediately deep-frozen in OCT compound.

Immunological reagents

The monoclonal antibodies (MAb) to the $\alpha_{1,2,3,4,6}$ and β_1 subunits used in this study are listed in Table 1. The serum of a patient suffering from bullous pemphigoid (BP) (titre 1:640) was used to label the BP antigen. A rabbit polyclonal antibody to laminin (Institut Pasteur, Lyon, France) was also employed (dilution 1:50).

Table 1. Monoclonal antibodies used to identify the subunits of the VLA family

Antigen	CD	MAb	Species	Source
VLA β_1	CD29	K20	Mouse	Bernard*[14]
VLA α_1	—	TS2/7	Mouse	Hemler†[1]
VLA α_2	CDw49b	CLB-thromb/4	Mouse	von dem Borne**[15]
VLA α_3	—	J143	Mouse	Old‡[2]
VLA α_4	CDw49d	B-5G10	Mouse	Hemler†[16]
VLA α_6	CDw49f	GoH3	Rat	Sonnenberg***[3]

Code Number: *P104, **P73, ***P55 from the Fourth International Workshop on Human Leucocyte Differentiation Antigens (Vienna, 1989).

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‡Kindly provided by Dr L.J.Old (Memorial Sloan Kettering Cancer Center, New York, NY, U.S.A.).

The following secondary sera and detection systems were used: biotinylated horse anti-mouse IgG serum (Vector Laboratories, Burlingame, CA, U.S.A.); biotinylated rabbit anti-rat IgG serum (Vector); biotinylated goat anti-rabbit IgG serum (Vector); fluorescein–streptavidin (Amersham International, Bucks, U.K.) and avidin–biotin–peroxidase complexes (Vector) for immunofluorescence and immunoperoxidase staining on tissue sections and NaCl separated skin; fluorescein isothiocyanate (FITC)-conjugated $F(ab')_2$ rabbit anti-mouse IgG antibody (Dakopatts, Copenhagen, Denmark); FITC-conjugated $F(ab')_2$ rabbit anti-rat IgG (Sera-lab, Crawley Down, U.K.) and tetramethylrhodamine (TRITC)-conjugated goat anti-human IgG (Kirkegaard and Perry Laboratories, Gaithersburg, MD, U.S.A.) for double-indirect immunofluorescence (IIF) labelling on epidermal cell (EC) suspensions; 10 nm-sized colloidal gold-conjugated goat anti-mouse IgG antibody (GAM-G10, Janssen Pharmaceutica, Beerse, Belgium) and 10 nm colloidal gold-conjugated goat anti-rat Ig antibody (GARa-G10, Janssen) for immunoelectron microscopic (IEM) labelling on EC and Langerhans cell (LC) enriched EC suspensions.

Immunofluorescence and immunoperoxidase labelling on tissue sections

Frozen sections 5 μm thick were processed for immunofluorescence using a three step biotin–streptavidin–fluorescein (SF) technique or for immunoperoxidase using an avidin–biotin–peroxidase (ABPx) technique as previously described.[17,18] The anti-VLA MAb were diluted 1:200 in phosphate-buffered saline (PBS), except for TS2/7 which was diluted 1:50.

Sodium chloride separation of skin

For the study of α_6 expression, sodium-chloride-separated skin was also used. This was obtained by treating $2{\cdot}0 \times 10$ cm trimmed skin slices with 1·0 M NaCl.[19] Briefly, skin strips were immersed in 1·0 M NaCl and then slowly rotated for 72 h at 4°C. The epidermis was dislodged from the dermis by lateral traction and 1 cm^2 pieces were then deep frozen in OCT compound. Sections 5 μm thick were processed for immunofluorescence using antibodies to the α_6 subunit or to laminin using three step biotin–streptavidin–fluorescein technique.

EC suspensions and LC enrichment

Skin slices obtained using a keratotome were floated in a

0·125% trypsin solution in Ca^{2+} and Mg^{2+}-free PBS (Biochrom KG, Berlin, Germany) for 18 h at 4°C. The epidermis was separated from the dermis with fine forceps. The epidermal sheets were agitated in Hank's balanced salt solution (HBSS, Biochrom), supplemented with 10% heat-inactivated fetal calf serum (FCS) (Boehringer-Mannheim, Mannheim, Germany). Disaggregated EC were then filtered through gauze and washed twice in cold RPMI-1640 (Biochrom), supplemented with 10% decomplemented human AB serum. For LC enrichment, EC suspensions were subjected to density centrifugation using Ficoll-Hypaque (Pharmacia Fine Chemicals, Uppsala, Sweden). Cells from the interface were harvested and washed in supplemented RPMI. Both EC suspensions and LC-enriched EC suspensions were finally re-suspended in cold supplemented RPMI at a concentration of 1×10^6 cells/ml. The viability of EC was 85% or higher, as assessed by trypan blue exclusion.

Immunofluorescence labelling on EC suspensions

The EC suspensions were labelled with anti-VLA MAb, followed by the appropriate FITC-conjugates (dilution 1:40). After smearing on glass slides and air-drying, a second indirect immunofluorescence labelling was carried out using BP serum (dilution 1:50) and the appropriate TRITC-conjugate (dilution 1:50).

All incubations were performed in cold supplemented RPMI, and the same medium was used as the wash buffer.

Ultrastructural immunogold labelling

Immunolabelling for transmission electron microscopy (TEM) was performed on EC suspensions and LC-enriched EC suspensions. The cells were stained on ice. MAb to VLA subunits were added for 1 h. The cell suspensions were then washed three times with cold supplemented RPMI and centrifuged at 200 *g* for 10 min. The EC were further incubated for 1 h under slow rotation with the appropriate gold-conjugate (dilution 1:5). All reagents were diluted using cold supplemented RPMI. Finally, EC were fixed as a pellet with 1% glutaraldehyde in a Tyrode buffer at pH 7·4 and washed in the same buffer, post-fixed in 1% osmium tetroxide for 45 min, dehydrated in graded alcohols, embedded in Durcupan ACM (Fluka, Buchs, Switzerland) and then sectioned on a Reichert Ultracut E ultramicrotome. Thin sections were stained with uranyl acetate and lead citrate and examined with a Philips EM 400 electron microscope.

Controls

For all the above-mentioned immunostaining methods, the negative controls consisted of: (i) replacement of the first step MAb with mouse IgG of matched isotype (Chemicon International Inc., El Segundo, CA, U.S.A.) or normal rat serum (Chemicon); (ii) replacement of the MAb with the respective buffer solution. These were consistently negative.

Results

Normal adult human skin

The immunohistological reactivity pattern of MAb to VLA subunits on frozen sections of normal human adult skin is summarized in Table 2. The same pattern was observed either on immunofluorescence or with immunoperoxidase staining procedures.

MAb recognizing α_2, α_3 and β_1 subunits strongly labelled the cell membranes of the basal cell layer (Fig. 1 a–c). The staining was often more prominent along the apical–lateral surfaces of the basal cells (Fig. 1 a–c). The first suprabasal cell layer was focally labelled, although at a lower intensity. The staining of the suprabasal cell layer was mainly observed with MAb to α_3 and β_1. The peripheral germinative cells of the sebaceous glands and the outer cell layer of the eccrine excretory ducts were regularly stained. A faint labelling of the inner cell layer of the secretory part of both the eccrine and apocrine sweat glands was also observed. In the hair follicle, a regular labelling of the outer cell layer of the outer root sheath as well as of the germinative cells in the hair bulb was detected.

Table 2. VLA protein expression in adult human epidermis and adnexa

	α_1	α_2	α_3	α_4	α_6	β_1
Epidermis						
Horny layer	—	—	—	—	—	—
Granular layer	—	—	—	—	—	—
Spinous layer	—	(+)	+	—	—	+
Basal layer	—	++	+++	—	+++*	+++
Epidermal adnexa						
Hair follicles	—	++†	+++†	—	+++*	+++†
Sweat glands	—	++	+++	—	+++*	+++
Sebaceous glands	—	+†	+++†	—	+++*	+++†

*Linear staining at the BMZ.

†Labelling of the outermost cell layer.

Staining intensity: +++, very strong; ++, strong; +, present; (+), faint; —, not detectable.

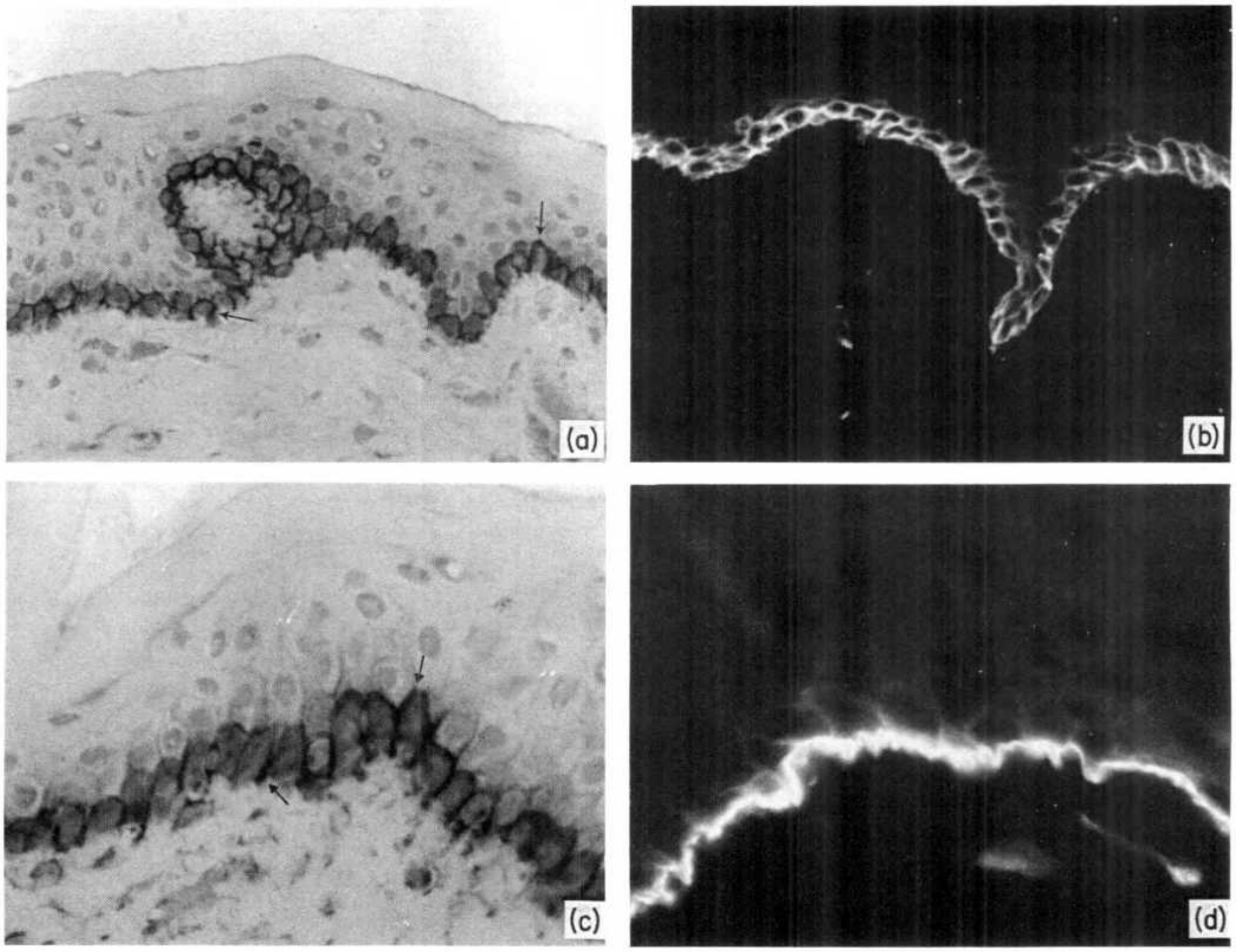

Figure 1. Expression of VLA proteins in normal human adult skin. The epidermal basal cell layer is intensely stained with MAb to the (a) α_2 (ABPx, × 250), (b) α_3 (SF, × 250) and (c) β_1 (ABPx, × 400) subunits. Note the accentuated labelling of the apical–lateral surfaces of the basal cells (arrows). (d) A strong linear labelling of the epidermal BMZ, together with a much fainter staining of the lateral surfaces of the basal cells is visible with MAb to the α_6 subunit (SF, × 800).

With the MAb to the α_6 subunit, a strong continuous linear staining of the BMZ was obtained at the level of the epidermis (Fig. 1d), the hair follicles, the sebaceous and sweat glands. The lateral cell surfaces of the basal cells in all these structures were also stained, although to a lesser extent (Fig. 1d).

With MAb recognizing the α_1 and α_4 subunits, no reaction was detected in the epidermis and adnexa.

Foetal skin

In foetal epidermis, MAb to α_2, α_3 and β_1 intensely stained the plasma membranes of the basal cell layer throughout the whole gestational period considered (Fig. 2a, b).

Immunoreactivity for α_2, α_3 and β_1 subunits was also present at the level of the developing hair follicles (hair germ, hair peg) then, from the bulbous hair peg stage on, it became restricted to the outer root sheath (Fig. 2b). Basal cells of the developing sebaceous and eccrine sweat gland were also stained (Fig. 2b). The α_6 subunit was expressed at the level of the BMZ in a linear fashion as early as week 10 (Fig. 2c). A similar labelling was then present around developing epidermal appendages. No labelling was observed in foetal epidermis with MAb to α_1 and α_4.

NaCl-separated skin

When IF was carried out on NaCl-separated skin using

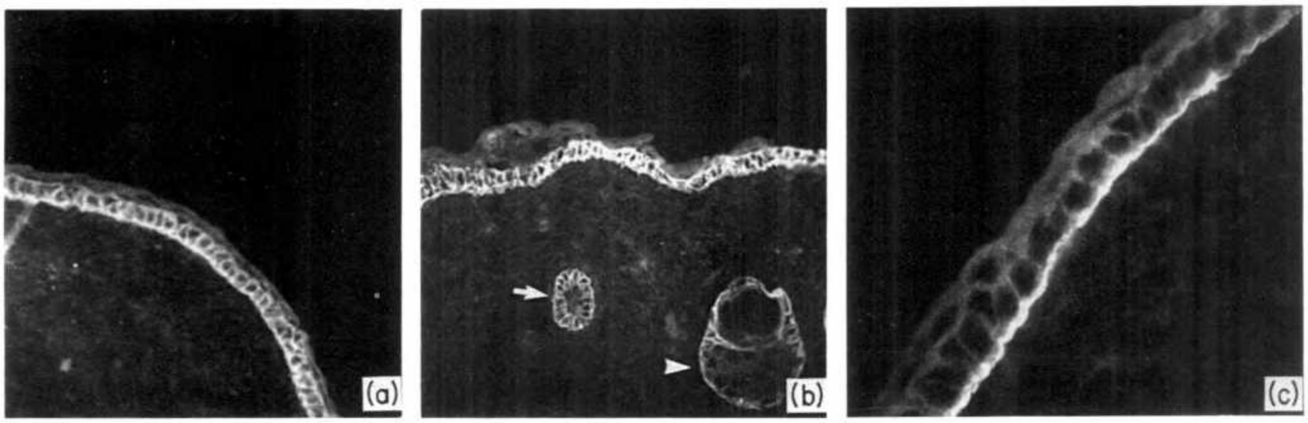

Figure 2. Immunofluorescence labelling of human foetal skin with MAb to the (a) α_2 (10 w GA, × 250), (b) α_3 (19 w GA, × 250) and (c) α_6 (10 w GA, × 800) subunits. The expression of α_2 and α_3 subunits is localized to the epidermal basal cell layer. A labelling of the outer root sheath of the hair follicle (arrow) and of the outermost cell layer of the sebaceous gland (arrowhead) is also evident. The α_6 subunit is expressed linearly at the BMZ level.

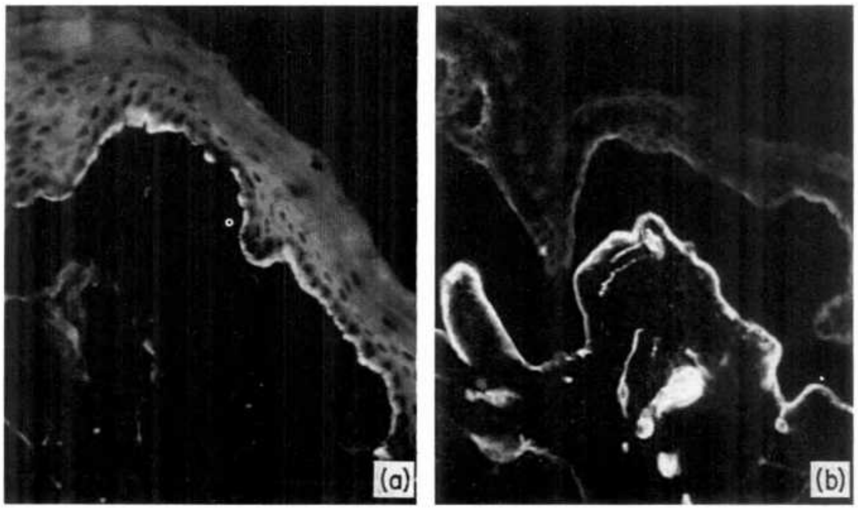

Figure 3. Immunofluorescence labelling of NaCl-separated skin with (a) anti-α_6 and (b) anti-laminin antibodies. The α_6 labelling is limited to the epidermal side of the separation, while laminin staining is present on the dermal side.

the MAb to the α_6 subunit, a sharp uninterrupted linear band of immunofluorescence was observed along the epidermal side of the separation, with no staining on the dermal side (Fig. 3a). In contrast, laminin staining was regularly detected on the dermal side of the separation (Fig. 3b).

EC suspensions

On EC suspensions, double IIF labelling using MAb to the various VLA subunits and BP serum showed that all the cells positive for the BP antigen, i.e. basal keratinocytes (BK), were also strongly reactive with MAb to α_2, α_3, α_6 and β_1 subunits. With anti-α_2, α_3 and β_1 MAb the fluorescence was uniformly distributed along the cell membrane, while with anti-α_6 MAb it was limited to one pole of the basal cells (Fig. 4a). The same pole of the BK was labelled with BP serum, thus demonstrating a strong expression of the α_6 subunit on the dermal pole of BK (Fig. 4b).

IEM labelling

Positive cells within the EC suspensions and LC-enriched EC suspensions were identified by the presence of gold particles scattered along the cell membrane. BK were identified as round-shaped cells with a high nuclear/cytoplasmic ratio and a cytoplasm that contained melanosomes and tonofilament bundles. Suprabasal keratinocytes were somewhat larger, had a lower nuclear/cytoplasmic ratio and more dense tonofilament bundles. LC were characterized by a villous surface, a notched

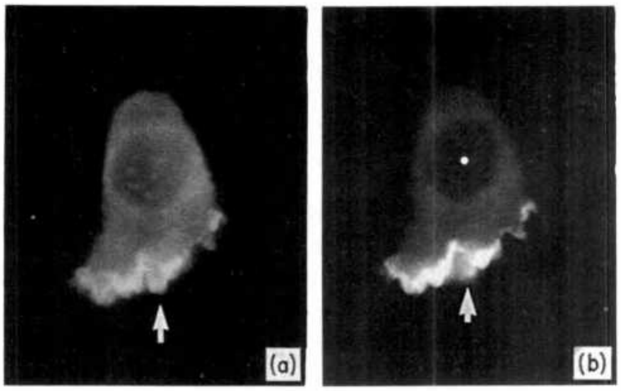

Figure 4. Double immunofluorescence labelling of EC suspensions. A cell positive for the BP antigen (a), i.e. a BK, is also α_6 positive (b). Note the localization of the labelling on the same pole of the cell (arrow) with both the BP serum and the anti-α_6 MAb (× 800).

nucleus and a relatively electron-lucent cytoplasm that contained characteristic Birbeck granules. Melanocytes were recognized as rounded cells with smooth contours, a rounded or slightly lobate nucleus and a relatively electron-lucent cytoplasm, with a well-developed Golgi apparatus and melanosomes.

BK were α_2, α_3 and β_1 positive, showing a high number of gold granules evenly distributed along the cell surface (Fig. 5). A few gold particles were present on the plasma membrane of suprabasal keratinocytes.

BK were also labelled with anti-α_6 MAb, but in this case a high density of gold particles was present on one part of the cell with only a few granules distributed on the rest of the cell membrane. When BK were longitudinally sectioned, the most strongly labelled part of the membrane was shown to correspond to their dermal pole (Fig. 6). Moreover, gold granules appeared to be mainly associated with hemidesmosomes (Fig. 7).

Keratinocytes were completely α_1 and α_4 negative.

With MAb to α_4, α_6 and β_1, a few gold granules were present on the plasma membrane of all Birbeck granules bearing LC (Fig. 8a, b), but with MAb to α_1, α_2 and α_3 no labelling was observed.

Several gold granules were evenly detected on the surface of melanocytes with MAb to α_3, α_6 and β_1 subunits (Fig. 9a, b). The same cells were consistently negative when the labelling was carried out using MAb recognizing the α_1, α_2 and α_4 subunits.

Discussion

The VLA proteins have a wide distribution, being expressed on cells of ecto-, endo-, and mesodermal origin. In normal human epidermis their common β_1 subunit has been shown to be present on BK, but also on LC and melanocytes.[20–25]. The results of our study show

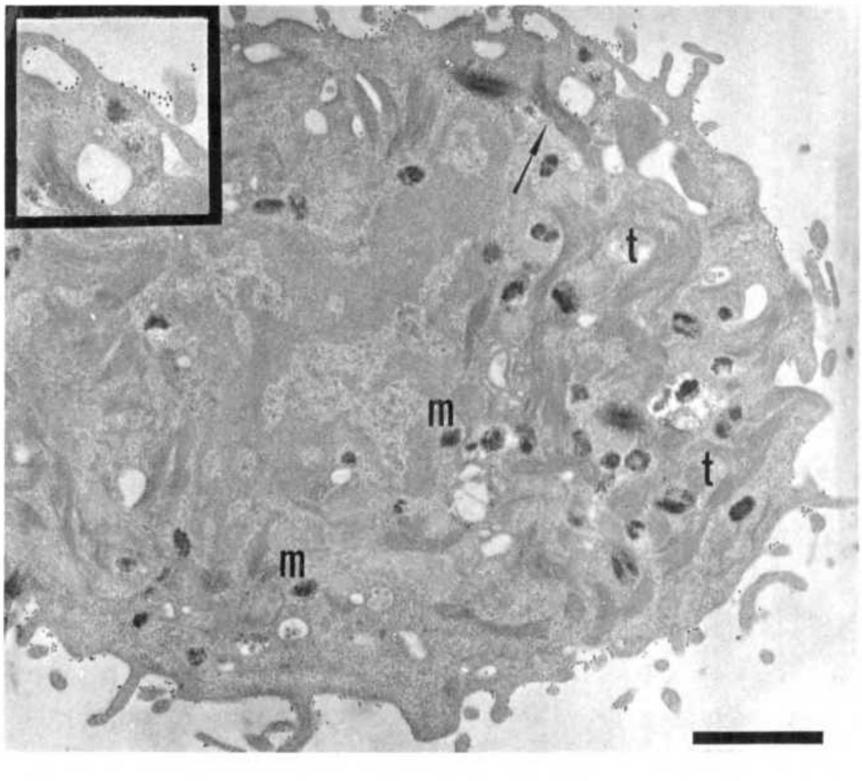

Figure 5. Immunogold labelling of EC suspensions with anti-α_2 MAb and 10 nm gold particles. Numerous gold granules are distributed along the cell membrane of a BK. m, melanosomes; t, tonofilaments. Inset: a higher magnification, showing the gold particles on the cell membrane (TEM, bar = 1 μm).

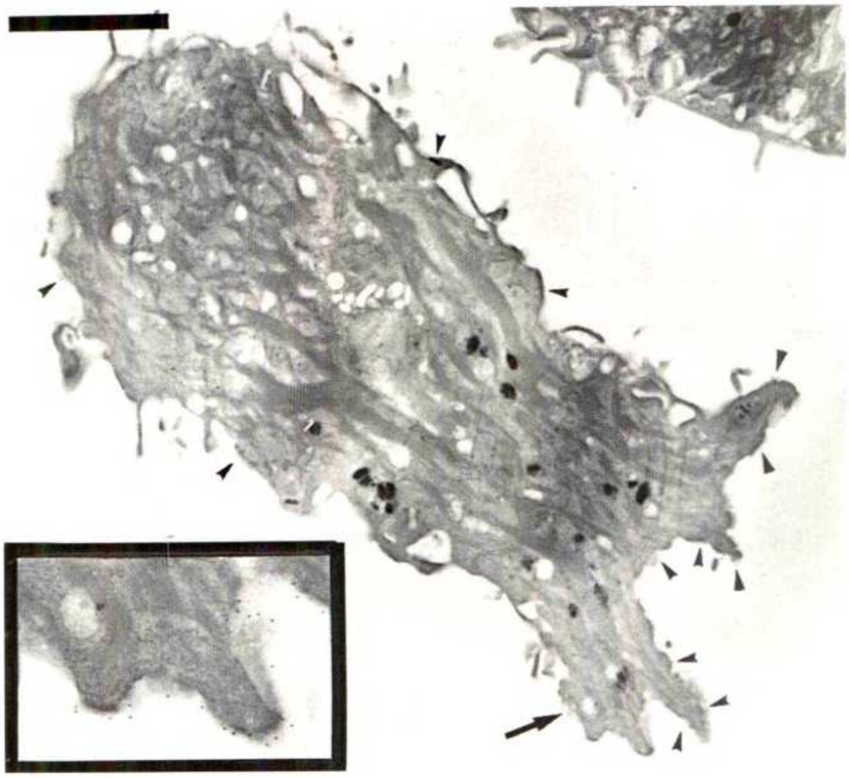

Figure 6. Immunogold labelling of EC suspensions with anti-α_6 MAb and 10 nm gold particles. Several gold granules (arrow) are present on the dermal pole of a longitudinally sectioned BK, while only a few granules (arrowheads) are distributed on the rest of the cell membrane. Inset: a higher magnification of the dermal pole showing the gold particles on the cell surface (TEM, bar = 2 μm).

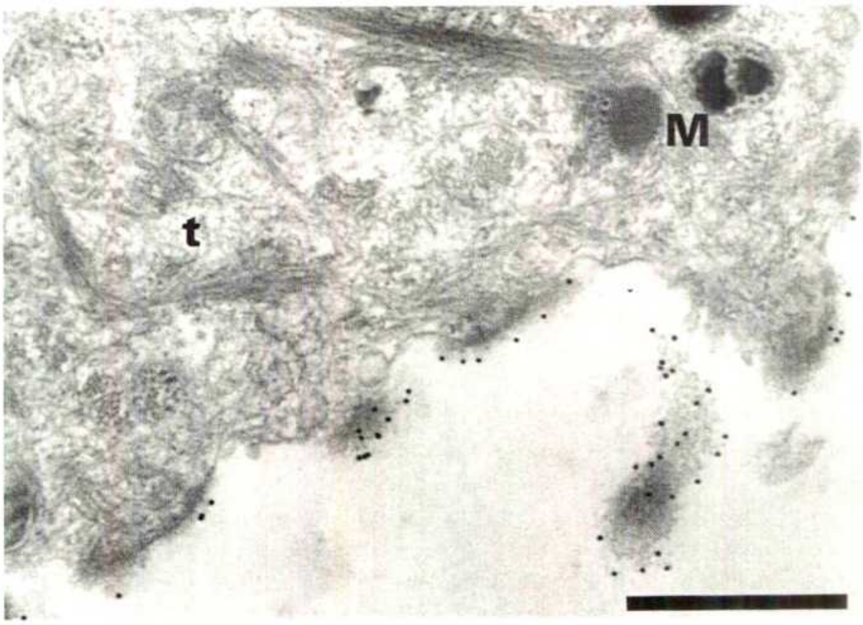

Figure 7. Immunogold labelling of EC suspensions with anti-α_6 MAb and 10 nm gold particles. On the membrane of BK gold granules are mainly localized in correspondence to the desmosomal structures. m, melanosomes, t, tonofilaments (TEM, bar = 0·5 μm).

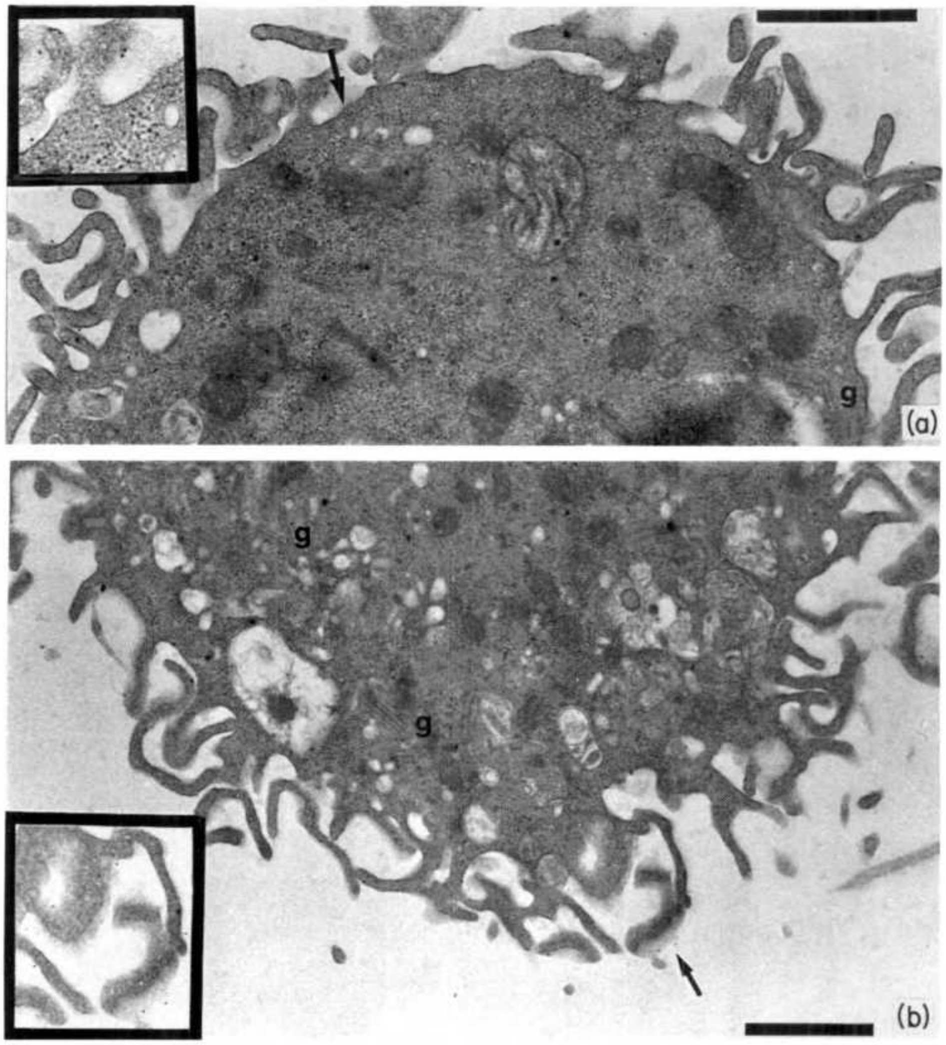

Figure 8. Immunogold labelling of LC-enriched EC suspensions with (a) anti-α_4 and (b) anti-α_6 MAb and 10 nm gold particles. A few gold granules are scattered along the plasma membrane of two LC. g, Birbeck granule. Inset: higher magnification, showing the gold particles on the cell surface (TEM, bar = 1 μm).

that keratinocytes express the α_2, α_3 and α_6 subunits, LC express α_4 and α_6 and melanocytes express α_3 and α_6.

The $\alpha_1\beta_1$ integrin does not seem to be present on any epidermal cell type. Among haematopoietic cells, VLA-1 is found only on long-term activated T-cells. Its reactivity in tissues seems to be limited to blood vessels, smooth muscle and fibroblasts in various organs.[1] In view of this very restricted *in vivo* distribution, the lack of VLA-1 expression on epidermal cells is not surprising.

Our results, show that $\alpha_2\beta_1$ and $\alpha_3\beta_1$ integrins are mainly present on basal keratinocytes, with a markedly decreased expression in the suprabasal cell layer, and are in agreement with other recent reports.[20,22,26] VLA-2 and -3 are known to represent receptors for the extracellular matrix components, collagen, fibronectin and laminin.[7] Since laminin and type IV collagen are two major components of the BMZ, the predominant localization of VLA-2 and -3 on BK may provide stability for the epidermal–dermal interface.

Interestingly, the staining for α_2, α_3 and β_1 on tissue sections often appeared more prominent on the apical–lateral rather than on the basal surfaces of BK. This finding was not confirmed by the IEM labelling of EC suspensions, where α_2, α_3 and β_1 were evenly expressed on the entire cell membrane of BK. This fact may be explained either by a redistribution of these transmembrane glycoproteins or by the revealing of blocked or cryptic antigenic sites of plasma membrane during the preparation of EC suspensions. Therefore, other techniques such as *in situ* IEM or laser confocal microscopy

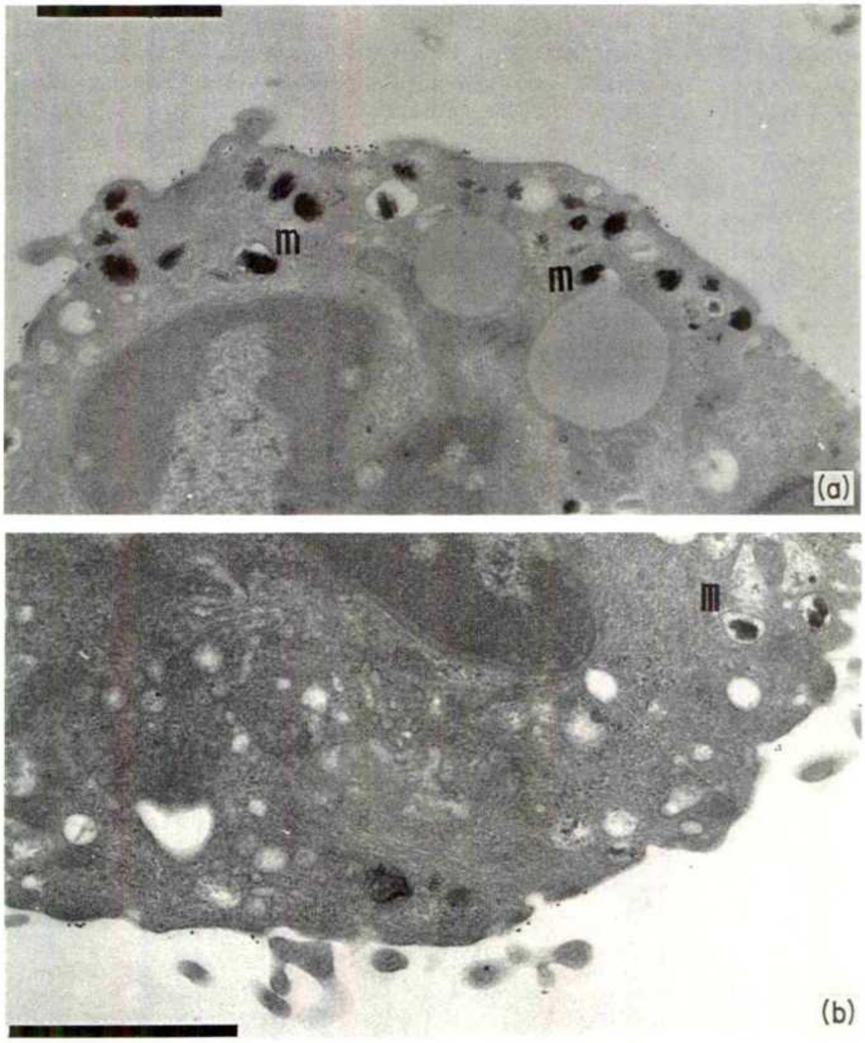

Figure 9. Immunogold labelling of EC suspensions with (a) anti-α_3 and (b) anti-α_6 MAb and 10 nm gold particles. Some gold granules are distributed along the plasma membrane of two melanocytes. m, melanosomes (TEM, bar = 1 μm).

are needed to establish the precise *in vivo* distribution of VLA-2 and -3 on the cell membrane of BK. A significant proportion of these antigens is always present on the apical–lateral surfaces of BK, i.e. in areas of cell–cell contact.

A role for some VLA proteins has been recently suggested, not only in cell–matrix but also in cell–cell adhesion processes. The preferential localization of VLA-3 at cell–cell contact sites in culture, for example, suggests its involvement in intercellular interactions.[27] The ability of MAb to the β_1 subunit to dissociate colonies of cultured keratinocytes without any effect on cell–substrate adherence indicates a cell–cell adhesion function of β_1 integrins.[28] Thus, VLA-2 and -3 could be involved in maintaining the adhesion among BK. When BK move to the suprabasal layer and begin to differentiate, the expression of VLA-2 and -3 declines rapidly, suggesting a function limited to the basal cell layer.

The α_6 subunit is also strongly expressed on BK, with a characteristic accentuated staining at the BMZ level. With both LM and EM, our observations on EC suspensions clearly demonstrate that α_6 expression is polarized on the dermal pole of BK. On platelets, VLA-6 has been identified as a laminin receptor.[29] Laminin is localized to the lamina lucida of the BMZ, in close contact with the plasma membrane of BK, and is thought to play a role in epithelial cell attachment to the basement membrane.[30] Expression of α_6 on the dermal pole of BK strongly suggests that this integrin mediates the anchorage of epidermal basal cells to the BMZ, possibly by binding to laminin. While the α_6 subunit is bound to the common VLA-β_1 polypeptide on haematopoietic cells, on normal and malignant epithelial cell lines it has recently been shown to be associated with a β_4 subunit.[11] The different labelling pattern observed on BK with MAb to α_6 and β_1 indirectly argues in favour of the presence of $\alpha_6\beta_4$

heterodimers on the basal pole of BK. It is tempting to speculate that on BK two groups of VLA proteins, characterized by two different expression patterns, are present: $\alpha_6\beta_4$, mediating basal cell anchorage, and $\alpha_2\beta_1$ and $\alpha_3\beta_1$, also mediating intercellular adhesion.

The preferential localization corresponding to hemidesmosomes of α_6 remains, at present, unexplained. Nevertheless, the association between another component of the dermal–epidermal junction, the BP antigen, and hemidesmosomes should be remembered.[31]

Treatment of human skin with 1·0 M NaCl is known to separate the BMZ at the level of the lamina lucida, leaving the BP antigen on the epidermal side and laminin on the dermal side.[19] As labelling with MAb to α_6 is limited to the epidermal side of this substrate, the superficial localization of α_6 within the BMZ is more clearly demonstrated.

In foetal skin, α_2, α_3 and α_6 subunits were expressed on BK by 10 weeks EGA, the youngest tissue examined. This finding is not surprising considering that type IV collagen and laminin are present in the dermal–epidermal junction as early as 5 weeks GA.[32]

Among other epidermal cell types, LC showed clear, although weak, α_4 and α_6 positivity, using immunoelectron microscopy. Our failure to detect α_4 and α_6 reactive epidermal dendritic cells on tissue sections could be explained by the low number of exposed antigenic sites, which may, therefore, escape detection.[33] Moreover, as we have previously shown by double-IIF labelling and by IEM on EC suspensions, LC express the common VLA β_1 chain.[25] Hence, the presence of one or more VLA-α subunits on LC is not surprising. Among leucocytes, the α_6 chain is strongly expressed on platelets but also, to a lesser extent, on monocytes and on monocytoid cell lines, including, as our study shows, epidermal LC.[11] The α_4 subunit expression on bone-marrow-derived LC is in keeping with its presence on most mononuclear blood cells. On lymphocytes, dual functions have been attributed to VLA-4 protein, namely cell adhesion to fibronectin and to other cells.[34] Similar functions in guiding the migration and localization of LC or in mediating LC adherence to other cells, such as lymphocytes, could be hypothesized. However, the role of the α_4 and α_6 subunits on the membrane of LC remains to be elucidated.

As far as we know, the present study is the first to demonstrate the selective expression of two α subunits of the VLA family (α_3 and α_6) on normal human melanocytes. To date, preliminary data show that melanoma cells in culture[35] or *in situ*[36] express adhesion molecules of the integrin superfamily. Further investigation of the expression of VLA proteins on melanoma cells as compared to their normal counterparts could help to define more clearly the altered adhesive and migratory properties of these malignant cells.

In conclusion, our study shows that epidermal cells (keratinocytes, melanocytes, LC) express VLA proteins in a cell-type specific pattern. It seems very likely that, as in other tissues, VLA proteins play an important role in the cell–cell and cell–BMZ interactions in human epidermis. The precise involvement of VLA proteins in these processes, as well as their modification in disease states, require further investigation.

Acknowledgments

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Seasonal changes in human hair growth

VALERIE A.RANDALL AND F.J.G.EBLING*
Department of Biomedical Sciences, University of Bradford
**Sub-Department of Dermatology, Academic Division of Medicine, University of Sheffield, Royal Hallamshire Hospital, Sheffield, U.K.*

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Summary Various parameters of hair growth were determined every 28 days for 18 months in 14 healthy Caucasian men aged 18–39 with indoor occupations in Sheffield, U.K. (latitude 53·4°N). In the scalp the proportion of follicles in anagen reached a single peak of over 90% in March, and fell steadily to a trough in September. The number of shed hairs reached a peak around August/September, when least follicles were in anagen. At this time the average loss of hairs was about 60 per day, more than double that during the preceding winter. The rate of growth of the beard was lowest in January and February and increased steadily from March to July to reach a peak about 60% above the winter level. The rate of growth of thigh hair showed a similar pattern though with less pronounced differences. No seasonal fluctuations in finger- or toenail growth were detected.

The human species, *Homo sapiens*, has lost most of its body hair during the course of evolution. Conspicuous hair remains on the scalp, the axilla and pubic region and in the male beard. Elsewhere, except on the friction surfaces, hair follicles are present, but for the most part they produce only short and often barely visible hairs. Nevertheless, as in all other mammals the activity of all follicles is cyclical. Phases of activity or *anagen* are separated by phases of quiescence or *telogen* during which the fully grown hair remains anchored in the skin by an expanded base known as a club.[1,2] The club hair is shed after a new period of anagen has started. Scalp follicles may each remain active for several years, though more than 10% of them are resting at any particular time and 100 hairs or more may be shed each day from a single scalp.[3] In the thigh, in contrast, the mean duration of anagen has been estimated at only 54 days in males and less than half that in females.[4]

The cyclical activity of the follicle is the mechanism by which mammals change their pelage to meet the exigencies of growth, seasonal changes in the ambient environment and, perhaps, normal wear and tear.[5] Some species (for example, the Norway rat)[6] grow new hair and moult in a patterned sequence. In others, such as the guinea-pig, the moults are more diffuse.[7]

Moulting appears to be controlled by an interaction of endogenous and exogenous factors. On the one hand, follicles have an endogenous rhythm which continues even when they are transplanted to other sites or other individuals. On the other hand, their cycles can be markedly accelerated or retarded by systemic factors.[5] It seems likely that environmental cues, mainly the photoperiod but with some influence of temperature, mediate through the hypothalamo-hypophysial pathway the production of steroid and thyroid hormones and that these in turn influence the hair growth cycles. Human postpartum alopecia, which appears to be an interference with the hair cycle by hormones circulating in late pregnancy,[8] may be a legacy of this mechanism, as may diffuse alopecia linked with malfunction of the thyroid.[9]

Hair which is used for social or sexual communication, either as a visual signal or as a component of scent glands, is under separate control, for the adaptive reason that socio-sexual signals are a matter only for adults, not for immature animals still under parental care. Such structures are almost invariably under the influence of androgens;[10] the growth of human axillary and pubic hair in both sexes, as well as the male beard and any other conspicuous hair on the male trunk and limbs, and sometimes also on the female, is similarly androgen-dependent.

The fact that human hair follicles, just as those of other mammals, undergo cyclical activity and are influenced by hormones raises a question of considerable evolutionary interest as well as of practical importance in the assessment of pharmacological effects: does human hair growth fluctuate seasonally? A single report that three women in New York experienced maximum hair fall in November[11] suggests this possibility. To provide a definite answer, various parameters were determined over 18 months in 14 males.

Methods

Subjects

The subjects consisted of 14 healthy Caucasian men aged 18–39 years with indoor occupations in Sheffield, U.K. (latitude 53·4°N). With the exception of one highly-motivated engineer, they were scientists or technicians (i.e. a particularly reliable, highly trained study group). They each collected beard shavings, shed head hair, finger- and toenail clippings and recorded the number of hours spent outdoors. They were visited every 28 days for 18 months; at each visit hair samples were obtained, by the same scientist, from five areas of the scalp and the thigh, an androgen-sensitive area.

Methods had to be used which were both logistically feasible and likely to produce data which could be rigorously analysed. Moreover, it was essential that all procedures should be sufficiently non-invasive as to be repeatable and acceptable to the volunteer participants over a long period.

Scalp

Percentage of follicles in anagen. In the scalp, the proportion of follicles in anagen was assessed from the roots of plucked hairs. However, rather than sample hairs being pulled out as a mass from a single region, the so-called trichogram,[12] we plucked single, long hairs at random in each specific scalp area. Each hair was gripped with spade-ended forceps placed as close as possible to the skin and extracted by a sharp pull. Ten hairs were removed from the vertex, the left and right parietal regions, and the left and right occipital regions, making a total of 50 hairs for each subject at each examination. Hair-root status was assessed under the dissecting microscope.[13]

Shed hairs. Subjects collected all hairs shed during combing or brushing over the 4 days prior to each visit. In addition, they were instructed to shampoo the scalp on the penultimate day, retaining all detached hairs using a plughole sieve.

Hair diameter. The hair diameter for each hair was measured at 7 and 14 mm from the base of the plucked root after careful placing on its widest axis.

Beard

Subjects were asked to save all hair shaved from the face, by whatever method, during the 7 consecutive days immediately before each visit, and the samples were weighed. Wet shaved hair was first washed, filtered and dried.

Thigh

Percentage of follicles in anagen. At the first visit, a template was used to delimit an area of 40 × 40 mm on the ventral side of the left thigh, and the corners were marked using a ball-point pen. The total number of hair-bearing follicles was then counted, before the whole area was shaved with a safety razor. To enable repetitive examination of the same area, a height-measuring device was used to obtain vertical and horizontal co-ordinates for each subject. At the next examination the number of reappearing hairs was counted. Although this does not directly reflect the number of follicles active at the moment of examination, it is a reasonable measure of any changes over the period, as the inclusion of club hairs in follicles which have ceased activity will be at least partly offset by the omission of follicles which have started activity but not yet grown identifiable emergent hair.

Rate of growth. At the second and subsequent visits 40 hairs judged to be among the longest, and thus the most likely to have been growing for all or most of the period, were removed at skin level using curved scissors, and measured.[13] The area was then again shaved.

Hair diameter. The removed hairs were mounted in Canada balsam and their mean diameter measured.[14]

Nails

Subjects were asked to save all nail parings from fingers and toes.

Statistical procedures

The means and standard errors for each parameter for each month were calculated. In addition, the results were statistically assessed by non-parametric runs, turning points and χ^2-tests.[15]

Results

Outdoor activity

Despite their indoor occupations the subjects showed pronounced seasonal differences in their behaviour (runs test <0·01; turning points <0·001; phase length

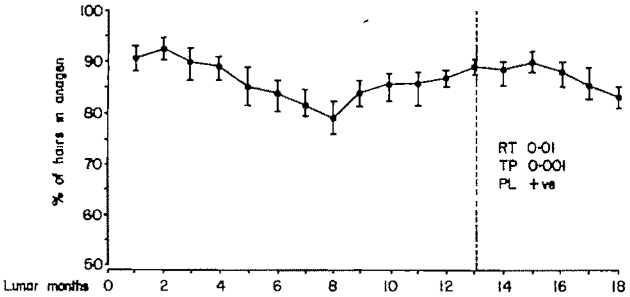

Figure 1. Monthly measurements of the percentage of plucked scalp hairs in anagen in 12 Caucasian men (50 hairs/subject/lunar month) in Sheffield, England (mean ± SEM). The samples from one subject were discontinued due to bleeding from each plucked follicle and no vertex samples were obtained from another.

positive), spending much more time outside during the summer than the winter (mean maximum *c.* 30 h/week in June/July compared to *c.* 11 h/week in January/February). The changes in outdoor activities closely paralleled the annual fluctuations in temperature and daylength.

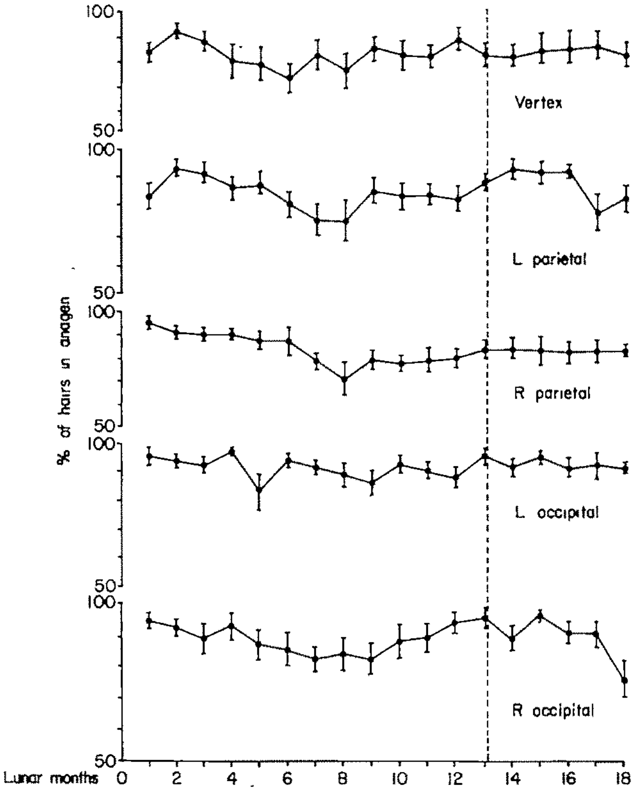

Figure 2. Percentage of hairs in anagen in five areas of scalp determined by random plucking of 10 hairs from each region each lunar month in 13 Caucasian men (mean ± SEM); vertex measurements were not included in one subject due to the beginning of male pattern balding.

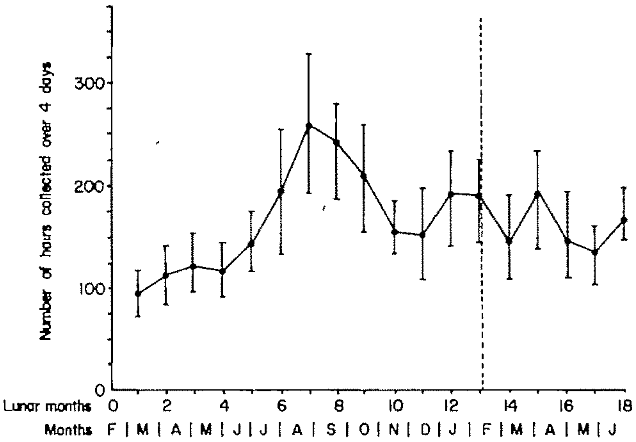

Figure 3. Number of hairs shed from the scalp in 14 Caucasian men over a 4-day period in each lunar month (mean ± SEM).

Scalp hair

The data show that, at high levels of statistical significance, the proportion of scalp follicles in anagen fluctuated seasonally (Fig. 1). It reached a single peak of over 90% around March, and fell steadily to a trough in September. The pattern appeared to have been manifested in each of the areas of the scalp, when considered separately (Fig. 2).

The numbers of shed hairs collected by the subjects closely followed the pattern of activity of the follicles (Fig. 3). Hair loss reached a peak around August/September, when the fewest follicles were in anagen. At this time, the average loss of hairs was about 60 per day, more than double that during the previous March, and compatible with the observed increase from 10 to 20% in the proportion of follicles in telogen.

The diameter of growing scalp hairs exhibited no significant seasonal fluctuations (Fig. 4) although the

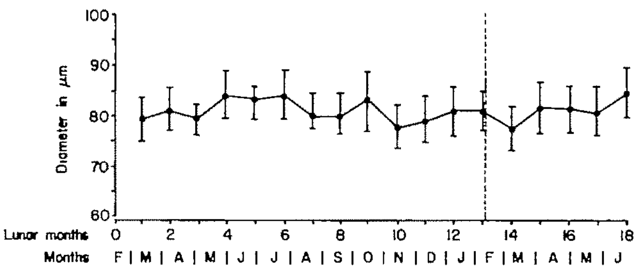

Figure 4. Diameter of growing scalp hairs from left parietal region of 13 Caucasian men (mean ± SEM).

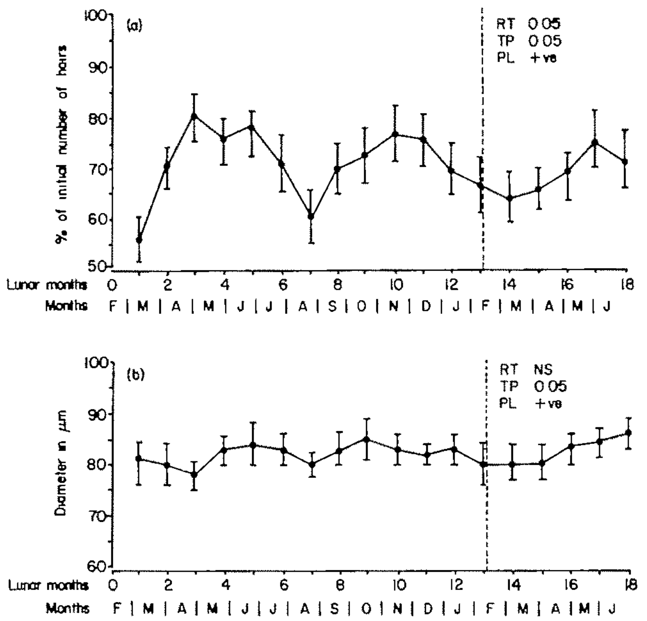

Figure 5. (a) Percentage of hairs in anagen on the thigh in 14 Caucasian men (mean ± SEM). (b) Diameter of thigh hair in 14 Caucasian men (mean ± SEM).

variations showed a similar pattern to that of thigh-hair diameter (Fig. 5).

Beard

The rate of beard growth (Figure 6a) also showed significant seasonal variation. It was lowest in January and February and from March it increased steadily to reach a peak about 60% higher in July than the winter level.

Thigh hair

The rate of growth of thigh hair (Fig. 6b) showed a similar seasonal pattern to that of the beard. The mean rate from February to March in the first winter was 0·27 mm/day. It then rose to reach a plateau of about 0·30 mm/day from June to September, and then declined steadily for 6 months. A similar rise occurred in the ensuing year.

The percentage of follicles in anagen (Fig. 5a) showed a remarkable and quite different pattern. It appeared to be lowest in March and August, and highest in May/June and November/December. The pattern is exactly what would be expected if the follicles were undergoing a spring moult and an autumn moult, in common with many other mammals of the temperate zone.

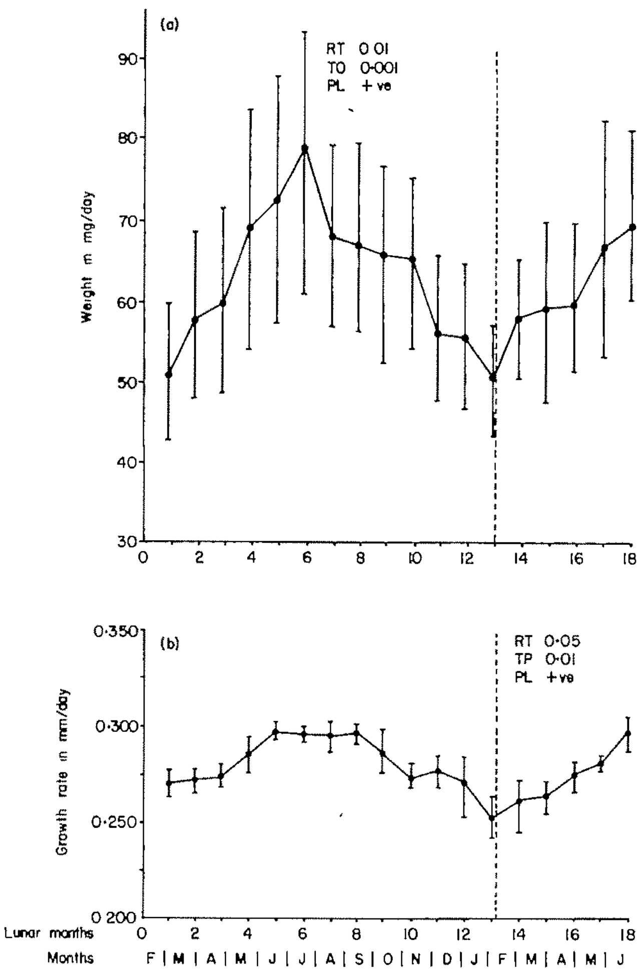

Figure 6. (a) Seasonal variation in beard growth in 14 Caucasian men (mean ± SEM) The wide standard errors reflect the normal variation in beard growth.[19] (b) Thigh hair growth rate calculated from samples removed every 28 days from a specific area of the left thigh from 14 Caucasian men (mean ± SEM).

The diameter of thigh hair (Fig. 5b) showed a similar pattern, though with less pronounced differences, which was just significant in some tests.

Nail growth

Neither finger- nor toenail growth, assessed by weighing all clippings obtained each month, showed significant seasonal fluctuations.

Discussion

The question of whether the circannual rhythms uncovered by this study have any evolutionary history or adaptive significance cannot be definitively answered. In the absence of direct evidence, the changes in the skin during the course of human evolution have been a matter of imaginative and often highly biased speculation. Moreover, no detailed studies of possible circannual changes in closely related hominids such as the chimpanzee or the gorilla have so far been reported.

The survival of hair on the scalp as distinct from the body is usually assumed to provide protection against the midday sun for a bipedal animal. So maintenance of the low winter level of hair shedding until the solstice and the postponement of hair fall until the end of summer might, perhaps, be postulated as having a selective advantage. Although the high incidence of male pattern baldness could be considered to weigh against this argument, the generally late onset of such balding may have put it outside the normal evolutionary pressures.

The evidence that the proportion of follicles in anagen in the thigh undergoes two seasonal troughs in each year is equally perplexing. It suggests that Man, in common with a number of other mammals, moults seasonally. In view of the sparsity of thigh and other body hair, even in males, this would not appear to have any selective advantage. If it were simply an evolutionary legacy, it would need to date back to a hairy ancestor, at least to the period when *Homo sapiens* was confined to the tropics, but perhaps by 3 million years if the argument is accepted that hair-loss began when hominids became upright.[16]

Seasonal changes in the rate of growth of the beard and the thigh hair may have a more ready explanation. Both types of hair are undoubtedly androgen-dependent, so the question arises whether the circannual changes could be related to androgen levels. Data from several studies indicate that the levels of plasma testosterone in European males do, in fact, fluctuate seasonally. Reinberg *et al.*[17,18] took samples every 2 months from five young males in Paris and found a peak in October. Smals *et al.*[19] took 3-monthly samples from 15 adult males in The Netherlands and reached similar conclusions. In April 1974 the mean was 666 ng/100 ml and the level rose significantly to 744 in July and 762 in October, before subsiding to 655 in January and 631 in April 1975. In Naples, at least, the circannual pattern becomes established prepubertally, even though the absolute levels are lower. Bellastella *et al.*[20] found the normal range of plasma testosterone in 106 healthy males aged 6–10 years to be 3–80 ng/100 ml. The mean values were lowest, under 50, in January and February, but rose to over 100 in July and August.

The evidence that the circannual pattern in plasma testosterone closely resembles those for growth of the beard and the thigh hair thus supports the view that these changes are related. Scalp follicles might similarly be influenced by hormones. The percentage in anagen is known to rise in pregnancy[8] and become reduced in pattern baldness.[21] As pattern baldness is, paradoxically, androgen potentiated[22] and androgen receptors have been demonstrated in cultured dermal papilla cells from scalp follicles[23], it is possible that the peak in hair fall, associated with an increase in the percentage of follicles in telogen in the scalp, is similarly related to testosterone levels.

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Prevalence of common and dysplastic naevi in a Swedish population

A.AUGUSTSSON, U.STIERNER,* M.SUURKÜLA AND I.ROSDAHL
*Departments of Dermatology and *Oncology, Sahlgrenska Hospital, Gothenburg, Sweden*

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Summary The naevus profile was examined in a Swedish population that was randomly selected from a census file. The participation rate was considered high at 82%. The number of common naevi (CN) and the prevalence of dysplastic naevi (DN) were investigated in 379 subjects (aged 30–50 years). The mean total body count of $CN \geqslant 2$ mm was 67 (range 1–300). As many as 22% of the population had 100 naevi or more and only 18% had less than 25. The counts were not influenced by age or sex. DN were diagnosed clinically in 18% (CI 14–22%) of the subjects and histologically in 8% (CI 5–11%). Subjects with dysplastic naevi had a significantly larger number of common naevi and a more sun-sensitive skin type than subjects without DN, $P<0{\cdot}001$.

The incidence and mortality rate of cutaneous malignant melanoma (CMM) are rapidly increasing in Caucasian populations all over the world. A lot of interest has been focused on sunlight as an aetiological factor but its exact role is still uncertain although several papers have reported an elevated risk of CMM after intermittent sun exposure or sunburn with painful blistering.[1] Further education to avoid the risks of sun exposure and sunburn is essential. As early diagnosis is the key to a good outcome of the disease, there is also a need for increased public and medical awareness of the early signs of malignancy. In addition to regular self-examination of the skin surface, screening programmes for individuals at risk have been suggested.

Several constitutional risk factors, including fair skin, red hair and blue eyes, have been accepted.[1,2] The strongest known risk factor is the presence of a large number of common naevi (CN).[3–7] Furthermore, there is a close association between dysplastic naevi (DN) and CMM in individuals with the dysplastic naevus syndrome.[8,9] Even in subjects with the sporadic form of DN, an increased melanoma risk has been reported.[5–7,10,11]

Further planning of screening programmes for high risk groups requires data on the frequency of common as well as dysplastic naevi in the general population. We have conducted the first Swedish population-based study of the prevalence of common and dysplastic naevi and their correlation with other constitutional factors.

Methods

Subjects

Five-hundred subjects, 30–50 years of age, were randomly selected from the census file in Gothenburg, Sweden. A letter was sent to each person offering a free skin examination by a specialist in dermatology. Thirty-one (6%) had either moved from the area or lacked a permanent address or were severely ill or deceased. Three-hundred and eighty-three (82%) agreed to participate although four of them were excluded as they were non-Caucasians. Of the drop-outs, 27 (6%) were interviewed by telephone but declined further participation, and 59 (12%) could not be reached. The participating 379 (183 men and 196 women) had a mean age of 42·5 years (range 34–52). The study was performed during the winter seasons 1986–88 and started with the oldest subjects.

Questionnaire

At the time of examination, the doctor and the patient together filled in a form. Ethnic origin and heredity for CMM were registered. The use of oral contraceptives was noted and an assessment of skin type according to Melski *et al.*[12] was made. Hair colour was rated according to a three-point scale as dark brown, light brown or red/blond. Eye colour was registered as brown/mixed, green or blue/grey.

Skin examination

All had a total body skin examination by the same

Correspondence: Dr A.Augustsson, Department of Dermatology, Sahlgrenska Hospital, S-41345 Gothenburg, Sweden.

dermatologist (A.A.). This included skin folds, palms, soles, scalp and the genital area. All brown macular or raised lesions ⩾2 mm considered to be pigmented melanocytic naevi were counted. Occasionally, it was difficult to differentiate between naevi, freckles or lentigines. If in doubt, the lesion was not counted. In randomly selected cases, independent mole counts were performed by one of the co-authors with excellent correlation.

The major clinical criterion for a dysplastic naevus was a diameter ⩾5 mm. In addition, at least two of the following criteria were required: an ill-defined or irregular border, speckled pigmentation, erythema or a pebbled surface.[13] A pigmented naevus ⩾10 mm and reported by the subject as appearing during the first year of life was considered to be a congenital melanocytic naevus (CMN). All patients with clinically diagnosed DN were offered excision of the lesions for histological examination. Some of them had a relatively flat common naevus excised at the same time to avoid bias in the histological diagnosis. All excisions were made by one investigator (A.A.) and one pathologist (M.S.) examined the slides without prior knowledge of the clinical diagnosis.

Pathology

The histological diagnosis of DN was based on the following features.

1. Irregular melanocytic hyperplasia (random single cells, streaks or interretal bridges) in the basal layer of the epidermis.
2. Little or absent nesting of the proliferated melanocytes.
3. Lentiginous acanthosis of the epidermis.
4. Stromal changes: subepidermal fibrosis, a non-specific inflammatory infiltrate and the occurrence of melanophages.
5. Additional possible features in the dermal component of a compound DN: the component is thin, inconspicuous and less horizontally extended than the epidermal component. The constituent cells have an immature 'lymphoid' appearance.
6. Cellular atypia of a significant degree (i.e. not exceeding what is generally seen in a lentigo maligna) was usually not seen.

The first two features listed were considered obligatory. The lesions had to demonstrate fairly cellular epidermal components to be properly judged according to the above criteria.[14,15] Lesions fulfilling only some of the criteria for the diagnosis of DN were registered separately as naevi with dysplastic features.

Statistical methods

Spearman's rank correlation test was used for the analysis of the data. For comparisons between groups, Wilcoxon's two-sample test was used. For comparisons of proportions between groups, Fisher's exact test was used. Trends in contingency tables were analysed using the Mantel–Haenzel χ^2-test.[16]. To estimate the probability of having a clinical DN as a function of rising number of common naevi, a maximum-likelihood estimation under order restrictions[17] was used.

Results

A total of 383 subjects were examined. Four were excluded as they were non-Caucasian. The mean total body count of CN ⩾2 mm was 66·9 (median 53, range 1–300). The numerical distribution of CN is shown in Figure 1. As many as 22% of the population had 100 CN or more.

There was no significant difference in the mean number of CN between men (70·8, range 4–300) and women (63·3, range 1–258). In the age-span studied (30–50 years), the number of CN was not influenced by age when analysed at 5-year intervals. We found no correlation between the number of CN and ethnic origin

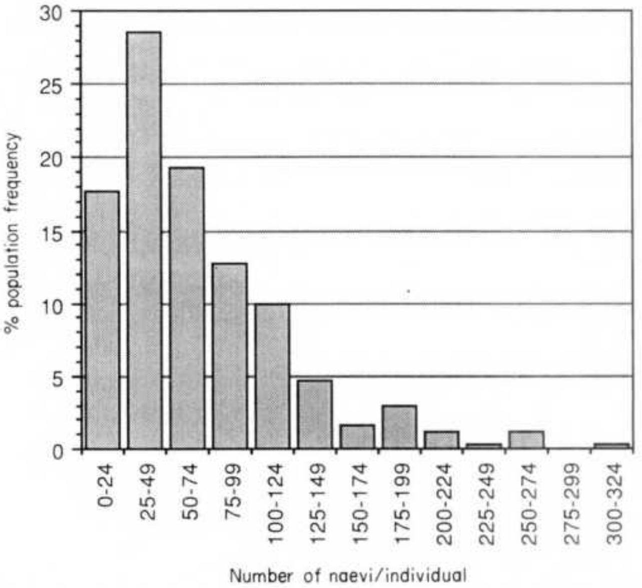

Figure 1. The numerical distribution of common naevi in a Swedish population ($n = 379$).

Table 1. Mean number of common naevi in relation to phenotype, ethnic origin, heredity for CMM and use of oral contraceptives

Factor	Number of subjects	Mean (SE) number of common naevi
Ethnic origin		
Scandinavian	351	67·4 (2·7)
Central European	28	60·8 (9·5)
Heredity for CMM		
Yes	14	79·3 (14·7)
No	365	66·5 (2·6)
Skin type		
I	2	25·5
II	28	63·0 (7 8)
III	277	69·0 (3 1)
IV	72	61·8 (5·8)
Hair colour		
Dark brown	59	53·3 (4·4)
Light brown	235	70·1 (3·6)
Red/blond	85	67·6 (5·0)
Eye colour		
Brown/mixed	77	63·7 (6·4)
Green	57	64·2 (5·6)
Blue/grey	245	68·6 (3·2)
Use of oral contraceptives		
Yes	116	65·1 (4·2)
No	62	62·0 (6·6)

(Scandinavian/Central European), heredity for CMM, skin type (I–IV), hair colour, eye colour or use of oral contraceptives (Table 1).

Heredity for CMM was found in 14/379 (3·7%). A congenital melanocytic naevus ⩾10 mm was noted in 22/379 (5·8%). Five out of 22 were ⩾20 mm, however no patient was found to have giant CMN. All the CMN seen were considered to be benign. Subjects with CMN did not differ from the rest of the population with respect to the number of CN or DN.

One or more clinical DN were found in 69/379, 18% (CI 14–22%). Sixty-five individuals agreed to excision of their DN and four declined the operation. All clinically identified DN were excised in 61 subjects; an additional four subjects had numerous DN and had from one to six of the more typical lesions removed. One had a melanoma *in situ* and was found to be a member of a family with the dysplastic naevus syndrome. Thirty of the 65 subjects had at least one naevus that fulfilled the histological criteria of DN and 16 had naevi with dysplastic features. The prevalence of histologically verified DN in this population was thus 30/379, 8% (CI 5–11%). In the 65 subjects operated upon, a total of 125 DN were excised. Of these, 45 were histologically DN and 24 exhibited dysplastic features. The remaining 56 biopsies were diagnosed as CN. Most DN diagnosed as CN histologically were clinically of the 'fried-egg type' and were less cellular than the rest of the lesions.

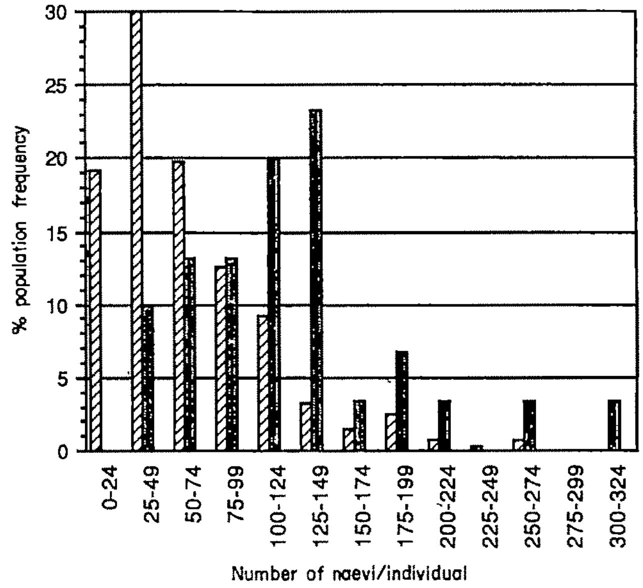

Figure 2. The numerical distribution of common naevi in subjects with and without histologically verified DN ▨, without DN ($n=349$); ■ with DN ($n=30$).

In 25/65 subjects one relatively flat CN was excised as a histological control. Five of these naevi showed dysplasia. All were small and the difficulty in differentiating between CN and DN microscopically increased with smaller size and less cellularity. The 30 individuals with histologically verified DN had a significantly larger number of CN [mean (SE), 118·8 (11·2)] than the rest of the subjects [62·5 (2·5), $P<0·001$]. Sixty-three per cent of the subjects with DN had more than 100 CN, while the corresponding figure for subjects without DN was 18% (Fig. 2). It is of interest to note that the 16 subjects with naevi that fulfilled the clinical criteria for the diagnosis of DN, but exhibited only dysplastic features histologically, had high counts of CN [mean (SE), 111·1 (13·8)]. This was significantly higher than counts in subjects without DN ($P<0·001$). Furthermore, even the 19 subjects with clinical DN diagnosed as CN histologically had significantly higher counts of common naevi [mean (SE), 80·7 (8·2)] than subjects without DN ($P<0·01$). In subjects with clinical DN, regardless of histology, the mean (SE) count of CN was 105·6 (6·7) compared with 58·3 (2·6) for the rest of the population ($P<0·001$) (Table 2). The 69 subjects with clinical DN had a more sun-sensitive skin type than those without ($P<0·001$). There was a

Table 2. Mean number of common naevi (CN) in subjects with and without dysplastic naevi (DN)

	n	Mean number of CN
No clinical DN	310	58
Clinical DN	69	106
Histological DN	30	119
Histological dysplastic features	16	111
Histological common naevi	19	81

highly significant trend towards a predominance for blond and red hair in this group ($P<0{\cdot}01$). However, they did not differ from the rest of the population in other respects such as age, sex, eye colour, ethnic origin or heredity for CMM.

Discussion

This is the first study of the frequency of common and dysplastic naevi in an adult Swedish population. Eighty-two per cent (383/469) of the subjects were examined. This is a high participation rate for a population sample selected from a census file. An additional 6% were interviewed by telephone, but declined further participation for various reasons. Twelve per cent also did not answer repeated letters. We found a mean total count of CN of 67, with a wide range of 1–300. Our impression from our clinics was that Swedes have more naevi than reported from other populations.[18–22] However, this figure was even higher than expected. It was surprising to note that as many as 80% had more than 25 naevi ⩾2 mm. The high mean mole count might be explained by the age-span studied. MacKie *et al.* have demonstrated high mole counts from 10 to 50 years of age with a peak in the group 20–29 years.[20] We selected the age range 30–50 years as most CN as well as DN have appeared at this age. In our population, we found a stable number of naevi when analysed at 5-year intervals. It is difficult to compare our results with previous reports from other countries. Very selected groups, limited skin areas and various types and sizes of melanocytic naevi have been examined. Nevertheless, earlier reports indicate a trend towards higher mole counts as melanoma has become more common.[22] Our high counts might be due to the fact that the whole body was examined. Furthermore, both dermal, compound and junctional naevi ⩾2 mm were counted. Precautions were taken not to misdiagnose other pigmented lesions as naevi. The same experienced dermatologist examined all subjects and the clinical diagnosis was in randomly selected cases verified by one of the co-authors.

To avoid the influence of sun-tan and freckles, the study was performed during the winter. If it was difficult to differentiate between naevi, freckles and solar lentigines, the lesion was not counted. The number of naevi did not differ between subjects with and without general freckling, indicating that freckles had no major influence on our counts. All the excised lesions, including the control biopsies were shown to be naevomelanocytic naevi on histology indicating that our high mole count was close to the true value.

We found a prevalence of clinically diagnosed DN of 18%. This is a high figure compared with a report of a population of selected Australian patients.[5] Cooke *et al.*, on the other hand, studied participants in a general community health survey and, despite the fact that large skin areas where DN are known to be frequent were excluded they reported a prevalence of 9%.[23] In a study of hospital patients in California the same clinical criteria as ours were used and a similar high prevalence of DN of 17% was found.[7] A large number of CN as well as the occurrence of DN are major constitutional risk factors for development of CMM. Our finding of an extremely high total mole count and a high prevalence of DN is in line

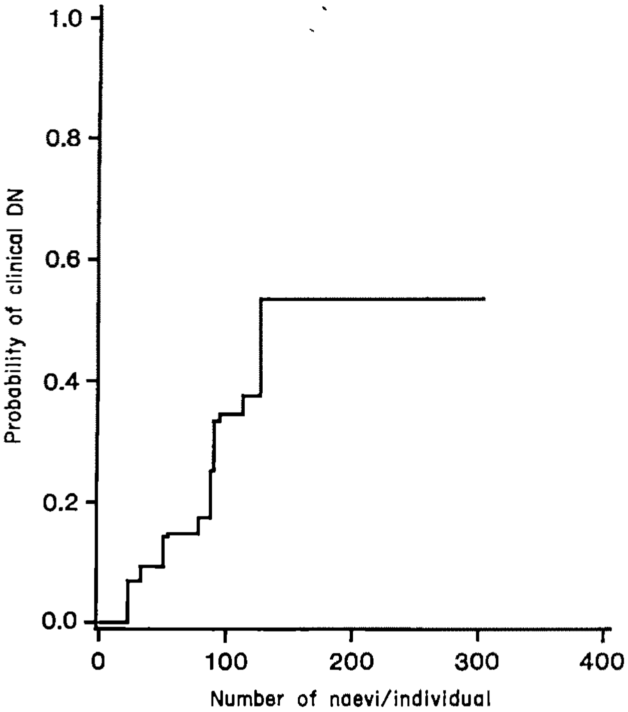

Figure 3. The probability of having a clinical DN with increasing number of common naevi.

with the fact that a population with a high incidence of melanoma has been studied.

It is interesting that subjects with clinical DN had nearly twice as many CN as those without. The risk of having at least one DN increases with the number of CN (Fig. 3). However, there was no correlation between the individual number of DN and CN. This excludes the possibility of a systematic error in the clinical judgement. In subjects with clinical DN, there was a trend towards blond and red hair colour and a more sun-sensitive skin type. A similar correlation with skin type has been reported.[24] In our material, the prevalence of histologically verified DN was 8%. We have only found one previous investigation on the prevalence of histologically confirmed DN. This study on California patients seen in a dermatology practice reported a figure of 4·9%.[25] The question remains whether clinically or histologically diagnosed DN is the most sensitive discriminator for identifying subjects at risk of developing CMM.

As more than half of this Swedish population has > 50 CN and close to one out of five has clinical DN, it is obvious that large-scale screening programmes will be too time-consuming and expensive. Furthermore, self-examination will be difficult to conduct. It is therefore necessary to define more detailed subgroups likely to develop melanoma. We are now analysing the results from a case-control study which deals with this question.

Acknowledgments

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Identification of the target antigen in chronic bullous disease of childhood and linear IgA disease of adults

FENELLA WOJNAROWSKA, P.WHITEHEAD,* I.M.LEIGH,* B.S.BHOGAL† AND M.M.BLACK†

Department of Dermatology, The Slade Hospital, Oxford, U.K
**Department of Experimental Dermatology, The London Hospital, London, U.K.*
†St John's Dermatology Centre, St Thomas' Hospital, London, U.K.

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Summary Disease-associated autoantibodies to basement membrane proteins have been used to characterize structural components of the epidermal basement membrane such as bullous pemphigoid (BP) antigen and epidermolysis bullosa acquisita (EBA) antigen (type VII collagen). The autoimmune bullous diseases characterized by IgA autoantibodies to the basement membrane zone (BMZ), i.e. linear IgA disease of adults (LAD) and chronic bullous disease of childhood (CBDC) may have circulating antibodies. Previous studies of tissue distribution and ultrastructural binding have suggested that the LAD and CBDC antigens are similar, if not identical, and differ from the target antigens of the other bullous diseases. We present the molecular characterization of the LAD/CBDC antigens by Western blotting of a large series of antisera. Seven of 33 sera (21%) were positive on immunoblotting and bound to the same antigen which has a molecular weight (MW) of 285 kDa. Using both defined polyclonal antisera to BP and LH 7.2 monoclonal antibody to type VII collagen (carboxy terminal) we have shown that the LAD and CBDC antisera both bind to an identical molecular weight protein which clearly differs from both the BP and EBA (type VII collagen) antigens. Although detectable in dermal tissue extracts like EBA, the MW of 285 kDa is heavier than type VII collagen (250 kDa, in our system, using non-collagenous standards). This study confirms the identity of LAD and CBDC antigens to be the same and to differ from previously described basement membrane proteins.

Chronic bullous disease of childhood (CBDC) and linear IgA disease of adults (LAD) are rare dapsone-responsive subepidermal blistering diseases, characterized by linear deposition of IgA at the basement membrane zone (BMZ) and a circulating IgA BMZ antibody in the absence of circulating IgG or C3 binding antibodies. This IgA autoantibody is found in the majority of sera from the patients with CBDC, but rarely in those with LAD. There is considerable clinical overlap between the two diseases, both with regard to a high incidence of mucosal involvement and a tendency to remission.[1] There is however a difference in the immunogenetics. In children with LAD 75% are HLA B8 positive, whereas this serotype is of normal incidence or is only slightly raised in the adults with LAD.[1,2] CBDC has long been regarded as a distinct entity.[3] However, this is not true of LAD, which was initially considered a variant of dermatitis herpetiformis (DH). This has now been differentiated from DH by the lack of gluten-sensitive enteropathy and the low incidence of HLA B8.[2]

LAD can be distinguished from the other subepidermal blistering diseases: bullous pemphigoid (BP), cicatricial pemphigoid (CP), pemphigoid gestationis (PG), and epidermolysis bullosa acquisita (EBA), by class of immunoreactant, the localization of the antibody binding site and the tissue distribution of the antigen.[4–6] IgA autoantibodies are unusual in bullous pemphigoid and are always found in association with other immunoreactants or antibodies. In addition the BP antigen is present in transitional as well as stratified squamous epithelia, whereas the LAD antigen is absent from transitional epithelia.[5] Split-skin studies using suction blisters[7] as substrate have demonstrated dermal as well as epidermal binding of antibody in LAD/CBDC. The majority of

Correspondence: Dr Fenella Wojnarowska, Consultant Dermatologist, The Slade Hospital, Headington, Oxford OX3 7JH, U.K

immunoelectron microscopy studies have shown deposition of antibody in the sublamina densa in LAD/CBDC[4,8-10] or a combined sublamina densa and lamina lucida pattern,[6,8,10,11] whereas in BP, PG and CP binding is generally accepted to be within the lamina lucida.[12-14] Although LAD/CBDC shares a lamina densa localization with EBA,[15] it is distinguished by epidermal binding on split skin.[16,17]

Immunoblotting and immunoprecipitation techniques have clearly defined the major bullous pemphigoid antigen as an epidermal glycoprotein of 220–240 kDa,[18] which has recently been cloned and sequenced.[19] A further 180 kDa epidermal antigen has been identified in BP[20,21] and PG.[22,23] Other minor epidermal antigens have been identified[21,24] but the relationship of these antigens to the classical BP antigen awaits molecular characterization. The antigen in CP remains controversial. The EBA antigen has been identified as the carboxy terminal of type VII collagen[25,26] in dermal extracts of human skin.

The purpose of this study was to identify the target antigen in both LAD and CBDC by immunoblotting and thus to resolve the controversy concerning the relationship of LAD to CBDC and to the other subepidermal blistering diseases. The monoclonal antibody LH7.2 to the carboxy terminal of type VII collagen was used as an internal standard to explore the relationship between LAD/CBDC antigen and type VII collagen as the patients tested had previously been reported as showing autoantibody binding to the lamina densa.

Methods

Patients and controls

CBDC/LAD patients. Thirty-four patients with an acquired subepidermal blistering disease in association with linear IgA deposition at the BMZ in clinically uninvolved skin, and a clinical response to dapsone were studied. The clinical details of many of these patients have been published[1] and are summarized in Tables 1 and 2. Sera from 18 patients with CBDC and 15 with LAD were studied. The sera had been characterized by immunofluorescence on intact and NaCl-split skin.[16,17] Some cases had also been studied by direct immunoelectron microscopy.[4] The results are summarized in the Tables. The sera was stored at −70°C.

Controls. Sera from 42 patients with BP, including one reference serum (with molecular weight 230 kDa, the gift of Dr John Stanley, National Institutes of Health,

Table 1. CBDC patients: clinical and immunopathological characteristics

		Site of antibody binding		
Patient	Age/sex	Immunoelection microscopy	NaCl split skin	Titre of IgA BMZ antibody
Positives				
1	16 F*	LD	E	1/4–1/64
2	10 M*	LD	E	1/8
3	5 M	LD	E	1/16
4	1 M		E	1/32
Negatives				
1	2 M		E	1/32
2	1 M		E	1/16
3	12 F		E	1/16
4	2 F		E	1/128
5	11 F*		—	—
6	8 F	LD	E	1/2
7	18 F*		E	1/128–1/256
8	7 F	LD	E	1/32–1/128
9	5 M		E	1/64
10	5 F		E	1/64
11	10 F		E	1/64
12	9 F		E	1/32
13	14 F*	LD	E	1/16
14	5 M		—	—

* Disease commenced before the age of 8.
—, negative; F, female; M, male; LD, lamina densa; E, epidermal binding; D, dermal binding. Blank indicates not tested.

Table 2. Adult LAD: clinical and immunopathological characteristics

		Site of antibody binding		
Patient	Age/sex	Immunoelectron microscopy	NaCl split skin	Titre of IgA BMZ antibody
Positives				
1	51 M	LD	E	1/64
2	55 F	—	—	—
3	53 F	LD	E	1/128
Negatives				
1	55 F		—	—
2	30 F	LD	—	—
3	76 F	LD	—	—
4	30 F		D	1/8
5	71 F	LD	—	—
6	14 F	LD	—	—
7	72 M	LD	E (1/8)	—
8	61 M	LD	E	1/2
9	47 F	LD	—	—
10	59 M	LD	—	—
11	72 F		D	1/2
12	49 F		D	1/8

—, negative; F, female; M, male; LD, lamina densa; E, epidermal binding, D, dermal binding; Blank indicates not tested.

Bethesda, MD, U.S.A.) and two with EBA (one reference serum with molecular weight 290 kDa, the gift of Dr R.A.Briggaman, University of North Carolina, Chapel Hill, NC, U.S.A.) were used as positive controls at a dilution of 1 : 5.

The monoclonal antibody LH7.2 to the carboxy terminal of type VII collagen which binds to a protein of MW 250 kDa was also used as an internal standard. Normal human sera from six healthy blood donors were used as negative controls.

Tissue preparation

Normal skin obtained from either breast or abdominal reductions, was cut into small fragments and incubated for 72 h in 1 M sodium chloride containing 50 μM phenylmethylsulphonylfluoride (PMSF) and 0·1 M ethylene diamine tetra-acetic acid (EDTA) at 4°C. The epidermis was then physically stripped from the dermis.

A basement membrane preparation was made from both the dermal and epidermal fragments by incubating them separately in 8 M urea containing 0·3 M mercaptoethanol and 50 μM PMSF and 0·1 M EDTA overnight (approximately 18 h). Tissue remnants were removed by filtration and the BMZ extract stored at −70°C until required.

Electrophoresis

Prior to sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE), samples were incubated in SDS sample buffer containing 10 mM TRIS pH 6·8, 100 mM dithiothreitol and 10% SDS at room temperature for 30 min. Electrophoresis was performed as described by Laemmli[27] using 5% PAGE.

The resolved proteins were transferred onto nitrocellulose as described by Towbin *et al.*[28] and the resultant blots blocked overnight at 4°C in phosphate-buffered saline (PBS) pH 7·4 (0·15 M sodium chloride, 0·02 M sodium phosphate) containing 3% bovine serum albumin (BSA) and 0·02% sodium azide.

Antisera

Once thawed, all sera were stored at 4°C in the presence of 0·02% sodium azide as was the monoclonal supernatant. Sera were tested neat or, immediately prior to use, were diluted 1 : 5 with PBS containing 0·05% Tween-20 (PBST). The monoclonal antibody supernatant was used undiluted.

Probing

Strips were cut from the blots and probed with primary antibody for 2 h at room temperature, excess antibody was washed off and then three sequential 10-min washes in PBST were carried out. The blots were then incubated for 1 h at room temperature with the appropriate peroxidase-conjugated second antibody, rabbit anti-human IgA (α chain) (DAKO Pats.) for the autoantisera or rabbit anti-mouse immunoglobulins (DAKO Pats.) for the mouse monoclonals. Both second antibodies were diluted 1 : 100 in PBST. Surplus antibody was washed off again by brief immersion in PBST with vigorous shaking followed by three sequential 10-min washes in PBST, finally the blots were rinsed twice in PBS.

Antibody binding was detected by immersing the blots in PBS containing 0·5 mg/ml diaminobenzidine and 0·02% hydrogen peroxide until sufficient colour had developed (approximately 15 min).

Results

Controls

The reference BP serum and nine of the other BP sera bound to the major BP antigen of MW 220 kDa in the epidermal extracts. EBA sera and LH7.2 all detected the carboxy terminal of type VII collagen at MW 250 kDa in the dermal extracts. The reference EBA serum, which had previously been shown to bind to a 290-kDa protein, bound at 250 kDa under our running conditions. Normal human sera showed no reaction with either the dermal or epidermal extracts.

CBDC/LAD sera

Immunoblotting of the CBDC sera showed that four of 18 sera from children reacted with a protein of 285 MW on

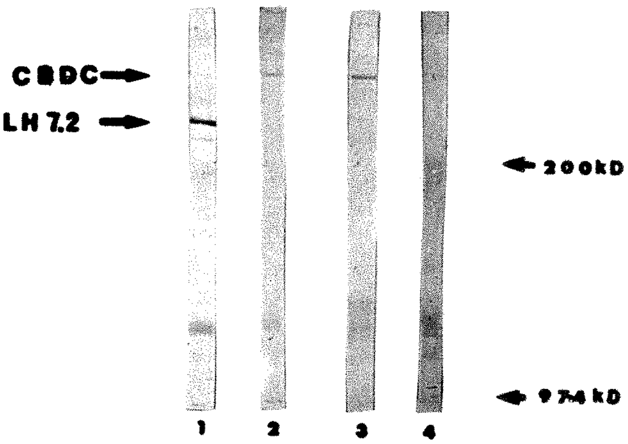

Figure 1. Immunoblotting of CBDC sera with dermal extracts. Lane 1: LH7.2, monoclonal antibody to collagen type VII blotting at 250 kDa. Lanes 2 and 3: CBDC sera blotting at 285 kDa. Lane 4: normal human serum.

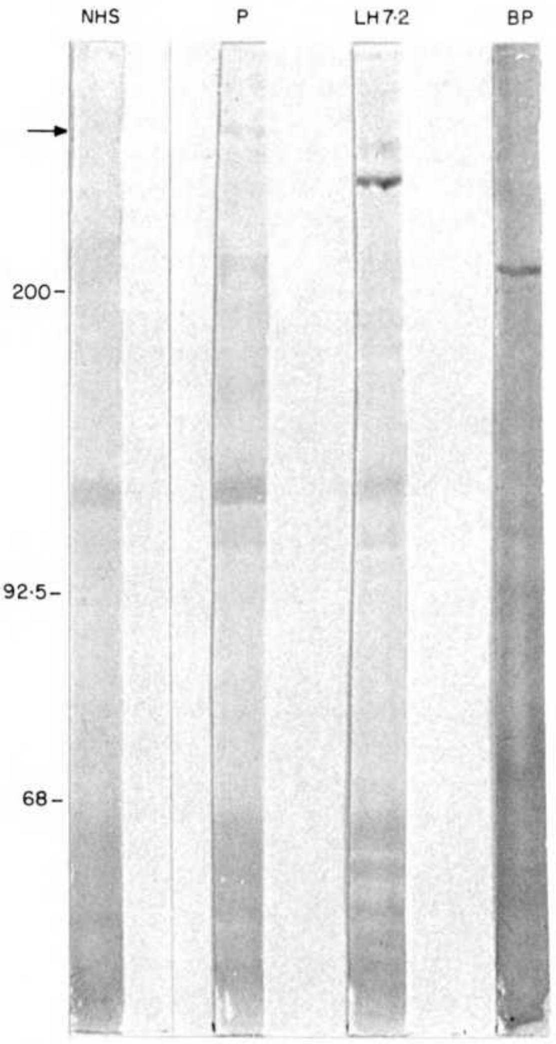

Figure 2. Immunoblotting of LAD sera. NHS, normal human sera; P2, LAD sera blotting at 285 kDa; LH7.2, monoclonal antibody to collagen type VII blotting at 250 kDa; all blotting on dermal extracts. BP, bullous pemphigoid sera blotting at 220 kDa with an epidermal extract.

dermal extracts of 1 M NaCl split skin (Table 1, Fig. 1). They showed no reaction with epidermal extracts. Three of 15 adults reacted with the identical MW band on dermal extracts only (Table 2, Fig. 2). There was a clear difference between the binding site of all the CBDC and LAD sera and the binding of LH7.2 to the carboxy terminal of type VII collagen which detected a band of 250 kDa under the prevailing gel conditions. As further confirmation of this different binding site, two well-characterized antisera to EBA antigen also binding to the carboxy terminal of type VII collagen showed binding at 250 kDa at a clearly different binding site to the positive CBDC/LAD sera.

Discussion

We have confirmed our preliminary report that the antigen for CBDC and LAD is identical[29] with a MW of 285 kDa. It can be detected only in dermal, not epidermal, extracts of human skin which accords well with the binding of these antisera to the sublamina densa on immunoelectron microscopy.[4] The CBDC/LAD antigen could be clearly distinguished from type VII collagen, which was detected at MW 250 kDa by the well-characterized monoclonal antibody LH7.2 and by two previously reported EBA antisera.[30] It could also be distinguished from the BP antigen which was found in epidermal, not dermal extracts, and at a lower MW of 220 kDa.

The number of positive CBDC and LAD sera was low at seven of 33 (21%) but there are a number of reasons for this. Many sera were negative by indirect immunofluorescence, even using split skin which increases the sensitivity of the testing,[17] and those sera which were positive usually had low titres. The technique of immunoblotting requires degradation of macromolecules by SDS-PAGE and this may damage conformationally sensitive epitopes. This problem could be addressed by immunoprecipitation where the antibody reacts with the native protein, however this requires the identification of a suitable cell producing the antigen (work in progress). The low level of positivity accords with other studies of BMZ diseases where only low titre antisera have been used and similarly low positive rates have been found.[23,24] Also, IgA antibodies are difficult to handle and blot poorly even when detected with IgA-specific second antibodies. Finally, we found that the serum reactivity diminished on prolonged storage or transport, which was unavoidable in many cases as retrospective sera were used due to the rarity of these diseases.

Although we confirmed our previous report of the identification of a target antigen of 285 kDa for CBDC and LAD, our findings differ from two other preliminary reports.[31,32] Zone *et al.*[31] studied 10 LAD patients and found using a split-skin technique that four had high titres of circulating BMZ antibody. These four sera reacted only with dermal extracts and immunoblotted at 97 kDa. We found only non-specific binding, present in normal human sera, BP sera and CBDC/LAD sera, at this molecular weight (Fig. 2). In a further study three patients with the sublamina densa pattern of IgA deposits on immunoelectron microscopy and dermal binding on split skin demonstrated binding to type VII collagen using a minigel system; this was confirmed in one case by two-dimensional electrophoresis.[32] Using

5% SDS-PAGE to enhance resolution of high-molecular-weight proteins, we were able to differentiate clearly the heavier molecular weight CBDC/LAD antigen from type VII collagen. Unfortunately we did not obtain positive blots with our three sera that had a dermal binding pattern on split skin. It may be that some LAD patients do have IgA EBA, but our LAD population has a different immunogenetic background to the American EBA patients.[1,33] Heterogeneity has been described for the BP antigen[24] and this may well apply to the CBDC/LAD antigen. However, the 285 kDa antigen seems to be the major target antigen for CBDC/LAD antibodies in our series from the U.K.

The presence of the CBDC/LAD antigen in dermal extracts clearly differentiates it from the epidermal-associated BP, CP, and PG antigens[18,20,22,23] which have been demonstrated only in epidermal extracts.

There is a difficulty in reconciling the biochemical extraction of dermal extracts together with lamina densa binding on immunoelectron microscopy with the observation of the majority of samples binding to the epidermal aspect of sodium chloride-split skin.[17] There was also a discrepancy in the binding of these antisera to suction blisters created in controls, where most bound to the dermal as well as epidermal surfaces in comparison with the 1M NaCl split skin.[7]

Previous studies have shown similarly mixed and contradictory results on immunoelectron microscopy. The majority showed binding to the lamina densa or sublamina densa,[4,8,10] some to the lamina lucida[8–10] and others have shown binding to both the lamina densa and lamina lucida.[6,11] These complex findings may reflect the spatial distribution of large BMZ molecules which does not necessarily correlate with the artefactual ultrastructural divisions into lamina densa and lucida. A clear comparison of immunoelectron microscopic distribution of antigens in skin split by different methods is now necessary as the level of cleavage may be subtly different. It is also clear that a number of lamina densa and sublamina densa antigens can be synthesized and assembled by keratinocytes in addition to fibroblasts, as has been shown for types IV and VII collagen. There is evidence that the expression of the LAD antigen in a skin-equivalent system requires fibroblasts in addition to keratinocytes,[34] suggesting a dermal contribution to the antigen. The BMZ proteins are not in a fixed rigid state but in a state of dynamic synthesis. Intracellular pools may be available which are exposed on BMZ injury whilst epitopes of more readily available antigens may be destroyed. Immunoblotting has been found to be more sensitive than other techniques and so may unmask dermal antigens. Despite these problems of ultrastructural localization our immunoblotting results were consistent and reproducible and represent the largest series of patients with LAD/CBDC investigated by immunoblotting.

This study has demonstrated that the target antigen in CBDC and LAD is identical, and that it is a unique antigen which is not involved in any of the other bullous diseases. It is interesting that this antigen is heavier than the other target antigens so far described, and this may explain the paradox of its lamina lucida and sublamina densa sites as such a large molecule could straddle both regions as has been described for laminin.[35]

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The mitogenic effects of sera from psoriatic subjects on normal dermal fibroblasts: an absence of correlation with the clinical activity of psoriasis

T.A.LAUBSCHER, N.HARRIS* AND D.O.CHALTON†
*Departments of Dermatology and *Clinical Science and Immunology,*
University of Cape Town/Groote Schuur Hospital, Cape Town, South Africa
Research Unit for Human Cell Biology of the South African Medical Research Council
†Institute of Biostatistics, Medical Research Council, South Africa

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Summary Serum was obtained from 21 normal and 22 psoriatic subjects, and the severity of skin disease in the psoriatic patients was recorded using the PASI score. The scores ranged from 1·8 to 51·0. The growth stimulatory effect of the sera on normal dermal fibroblasts in cell culture was assessed by measuring [^{3}H]-thymidine uptake. Each serum was assessed at four concentrations (2, 5, 10, 20%). The psoriatic sera were more growth stimulatory than normal sera, but this difference was statistically significant ($P<0{\cdot}02$) only at 20% serum concentration. Eleven of the 22 psoriatic serum samples had a mitogenic effect greater than the mean ± 2 SEM of all the normal sera; these sera were from patients with PASI scores of 4·5–51·0. Six of these psoriatic subjects were recalled after 5 months; their PASI scores were reassessed, and the mitogenic effect of new serum samples was compared with that of the initial samples. All of these patients displayed a change in serum mitogenic effect, but this was not consistent with the change in severity of skin disease over the corresponding time period. In one subject, the severity of the psoriasis had increased marginally over the 5 months, while the mitogenic effect of her serum decreased significantly (mean counts of 43808 vs. 32660; $P=0{\cdot}0029$).

Psoriasis is characterized by pathological features of hyperproliferation and inflammation involving keratinocytes, dermal fibroblasts and endothelial cells. While there is evidence of a humoral factor in the pathogenesis of psoriasis,[1] the nature or source of this factor remains unknown. Majewski *et al.*[2] reported that serum from subjects with psoriasis stimulated endothelial cell proliferation, and suggested that this mitogenic effect may play a role in the vascular abnormality of psoriatic lesions. There is evidence of dermal fibroblast hyperactivity in psoriasis,[3,4] but experiments comparing the mitogenic effects of sera from normal and psoriatic humans on fibroblasts have yielded conflicting results.[5–7] We studied the effect of normal and psoriatic sera on the incorporation of radiolabelled thymidine by normal skin fibroblasts, and investigated the possible relationship between the mitogenic effect of psoriatic serum and the clinical severity of the psoriasis. Thymidine uptake by cells *in vitro* is frequently used to assess cellular proliferation because the quantity of this nucleoside incorporated into growing cells is a reflection of DNA synthesis.[8]

Methods

Selection of patients: serum samples

Blood was obtained from 21 normal subjects (blood donors with no family history of psoriasis; 12 males, nine females). This normal human serum (NHS) was stored in 0·5-ml aliquots at −80°C; each frozen sample was thawed and used only once. The reference serum was obtained from a healthy 27-year-old female: 40 ml of this serum was frozen in 0·5 ml aliquots and these were used in all of the proliferation assays. The 22 psoriatic subjects (five males, 17 females) were selected from those attending Groote Schuur Hospital Dermatology Department for in-patient or out-patient treatment of chronic plaque psoriasis. Each patient was interviewed and examined by one of the authors (T.L.) and the severity and extent of psoriasis scored by using the Psoriasis Area and Severity Index (PASI).[9] Eighteen of the patients were

Correspondence: Dr T.Laubscher, Department of Dermatology, Groote Schuur Hospital, Observatory 7925, Cape Town, South Africa.

using either topical tar or topical corticosteroid preparations; four had received no treatment for 4–6 weeks. Eight patients commenced dithranol therapy after their initial assessments and serum sampling. Psoriatic human serum (PHS) (20 ml) was obtained from each subject and stored in 0·5-ml aliquots at −80°C. The controls and patients had a similar range of ages (18–86 years) with mean values of $35 \cdot 3 \pm 2 \cdot 3$ (SEM) and $40 \cdot 6 \pm 4 \cdot 4$ (SEM), respectively.

Isolation of fibroblasts

Normal skin was obtained from the forearm of a healthy 28-year-old male. The skin specimen was soaked in Dulbecco's modified Eagle's medium (DMEM) with 4 mM L-glutamine (Gibco) and 44 mM bicarbonate, containing 100 units/ml penicillin and 100 μg/ml streptomycin, for 2 h. It was cut into 1-mm pieces which were placed beneath sterile glass coverslips in tissue culture dishes. DMEM with antibiotics (as above) and 10% fetal calf serum (FCS; State Vaccine Institute, R.S.A.) (DMEM-10) was added to each dish and the cultures were incubated at 37°C in humidified 95% air/5% CO_2. This culture medium was renewed twice weekly.

After 4 weeks the cells were removed with 0·25% Trypsin (Difco) containing 0·02% EDTA and transferred to larger tissue culture dishes. The dead keratinocytes were removed, and the fibroblasts were grown in DMEM-10. The cells were subcultured weekly, and fibroblasts of 4–7th passage were used in the proliferation assays. Stocks of low passage cells were also frozen and stored in liquid nitrogen. After being thawed, cells were grown for 2 weeks before being used experimentally.

Proliferation assay

A suspension of fibroblasts in DMEM-10 was counted with a Coulter counter, and plated out into a 96-well tissue culture plate at a density of $1 \cdot 5 \times 10^4$ cells per well. Following a 30 h incubation, the DMEM-10 was removed, the cells were washed with sterile phosphate-buffered saline (PBS), and DMEM containing 0·2% FCS was added to each well for an incubation period of 65 h. On day 5 of the assay, 2, 5, 10 and 20% dilutions (by volume) of each human serum in DMEM were prepared. The DMEM with 0·2% FCS was aspirated, and 0·2 ml of each dilution of serum was added to a set of four wells. The reference serum, which was included in every assay, and five test sera (NHS or PHS) were analysed in a 96-well plate. Following an 18-h incubation, 0·4 μCi of [methyl-^{3}H]-thymidine (Amersham International) was added to each well, and the cells incubated for a further 4 h. DNA synthesis was determined by measuring the uptake of [^{3}H]-thymidine into trichloroacetic acid (TCA)-insoluble material. The fibroblasts were washed with PBS, fixed in cold 10% TCA and then washed with methanol. The cells were solubilized in 0·5% sodium dodecyl sulphate and added to scintillation fluid (Insta-gel; Packard Instruments) for scintillation counting. The radioactivity was expressed as disintegrations per minute (d.p.m.). The mean and the standard error of the mean (SEM) of the four replicate counts at each serum concentration were determined. All the NHS and PHS samples were evaluated once. Those PHS samples with a greater mitogenic effect (on comparison of initial NHS and PHS data) were re-assayed to confirm their hyperstimulatory nature.

Statistical analysis

The mitogenic effects of normal and psoriatic sera were compared by the method of least squares (as the split-plot design of the experiment was unbalanced) in order to eliminate the effect of variables such as different times of assay, different positioning of the sera in the 96-well tissue culture plates, and minor differences inherent in tissue culture systems. Using this method, mean counts at each serum concentration were obtained and significance testing was assessed by a modified *t*-test. An analysis of variance was performed on the data from hyperstimulatory psoriatic sera, in order to compare the mitogenic effect of the initial sample with that of the serum taken from the same subject 5 months later.

Results

The 22 psoriatic subjects in this study had psoriasis of varying severities. Their PASI scores ranged from 1·8 to 51·0, with 50% of the scores greater than 10·0. Two subjects had psoriatic arthropathy, and two displayed Köbner reactions on initial assessment.

The mitogenic effects of the normal and psoriatic sera on normal skin fibroblasts are shown in Figure 1. While there is a wide scatter of values for both sera, the results indicate that the stimulatory effect of PHS is greater than that of NHS. This difference is not consistent over the range of concentrations, and is statistically significant only at 20% serum concentration (i.e. *t*-value of 2·718 at $P < 0 \cdot 01$, using a modified *t*-test) (Table 1). For both types of sera the growth stimulatory effect was concentration dependent, with the stimulation of 20% serum concentration being four to five times greater than that

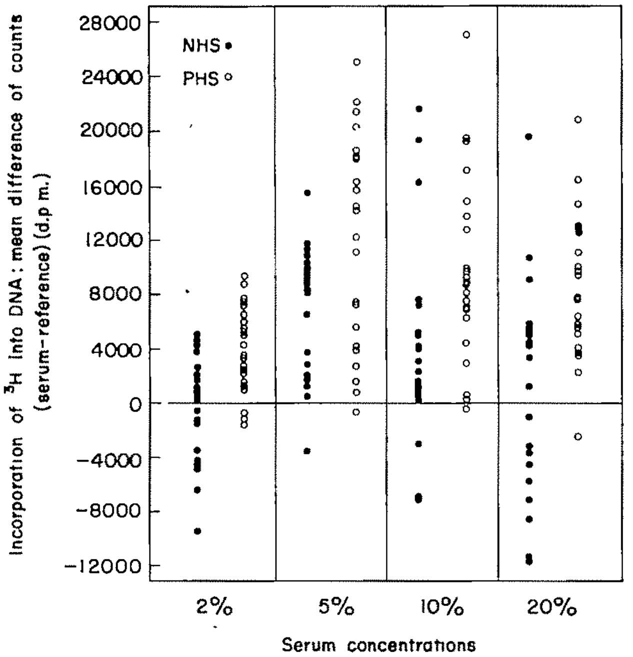

Figure 1. The effect of sera from normal and psoriatic subjects on DNA synthesis in skin fibroblasts. Sera were evaluated at concentrations of 2, 5, 10, 20% by volume, and compared with the reference serum. The data are presented as the difference between the mean counts of each serum (NHS or PHS) and the reference serum.

Table 1. Statistical results of mitogenic effects of normal and psoriatic sera on normal skin fibroblasts

	Concentration of serum			
	2%	5%	10%	20%
Mean difference of counts (serum–reference) (d.p m)	4681·7	5018·9	5450·3	8016·1
SEM	2949·1	2949·1	2949·1	2949·1
t-value	1·587	1·702	1·848	2·718

$P<0{\cdot}05$, $t=2{\cdot}002$; $P<0{\cdot}01$, $t=2{\cdot}666$.

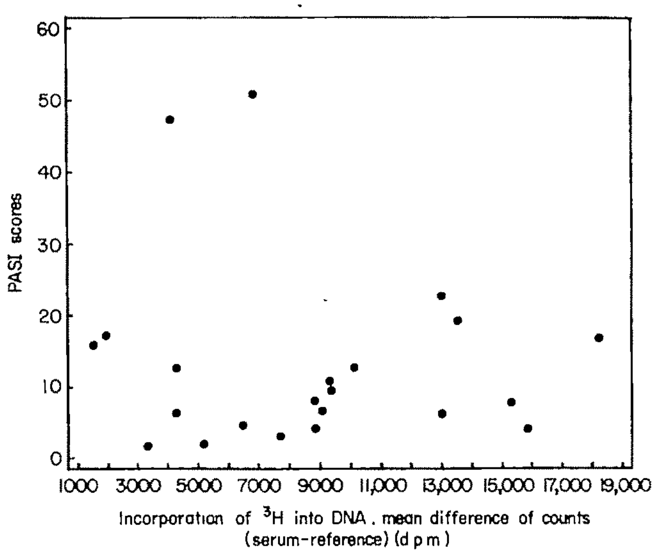

Figure 2. A plot of the individual PASI scores of the 22 psoriatic patients versus the stimulatory effect of their sera on [^{3}H]-thymidine incorporation by cultured dermal fibroblasts. The latter values are presented as the difference between the mean counts in the presence of the psoriatic serum and the reference serum. The experiments were performed over a range of serum concentrations (from 2 to 20%). This data represents the mean of all concentrations ($r=-0{\cdot}1358$, $P=0{\cdot}5466$).

of 2% serum concentration (results not shown). There was no apparent correlation between the PASI scores of the psoriatic patients and the stimulatory effect of their sera ($r=-0{\cdot}1358$, $P=0{\cdot}5466$) (Fig. 2).

Eleven (50%) of the PHS samples displayed a mitogenic effect greater than the mean +2 SEM of all the NHS samples. These sera were from subjects whose PASI scores ranged from 4·5 to 51·0. Serum samples, obtained from six of these psoriatic subjects (same PASI score range) 5 months later, were assayed simultaneously with their initial samples in order to assess any change in the stimulatory effect of each subject's serum. Table 2 compares the changes in the mitogenic effects of these pairs of serum samples with the corresponding

Table 2. The change in clinical severity (PASI score) of psoriasis in six subjects after a 5-month interval, with the corresponding change in mitogenic effect (mean counts) of each individual's serum on dermal fibroblasts. (Reference serum mean count was 30720)

	PASI scores		Incorporation of [^{3}H]-thymidine into DNA: mean counts (d.p.m.)						
Subject	April/May	Aug./Sept.	April/May	Aug/Sept.	Difference	*t*-value	Probability	95% confidence interval	
P6	51·0	5·3	31923	34560	2637	0·82	0·4229	−4143	9417
P17	18·1	13·5	34926	39450	4525	1·40	0·7830	−2255	11305
P19	4·5	4·0	40006	38698	−1308	−0·41	0·6866	−8088	5472
P20	7·8	8·4	43808	32660	−11148	−3·45	0·0029	−17928	−4368
P21	13·1	16·4	37914	34132	−3782	−1·17	0·2573	−10562	2998
P22	8·2	9·9	45674	44426	−1248	−0·39	0·7011	−8028	5532

changes in the PASI scores. Subject P20 was the only patient to show a significant reduction in the mitogenic activity of her serum (i.e. mean counts of 43,808 vs. 32,660; $P = 0{\cdot}0026$), despite increased severity of skin disease over the corresponding time period. In subjects P6 and P17 there were marked reductions in their PASI scores, while the stimulatory effects of their sera increased marginally.

Discussion

Psoriasis is a systemic disease with protean features, and while there have been suggestions of a pathogenic factor in the serum of patients there has been no attempt to investigate the possible relationship between the presence of this serum mitogenic factor(s) and the clinical types or severity of psoriasis. Priestley and Adams[5] demonstrated that sera from normal and psoriatic subjects had similar mitogenic effects on cultured dermal fibroblasts. However, others have since shown that the serum of psoriatic subjects has a greater growth stimulatory effect than that of control subjects.[6,7]

Our experiments have shown that the serum from psoriatic subjects had an enhanced ability to stimulate the uptake of a labelled DNA precursor by human dermal fibroblasts when compared with serum from normal subjects. However, this mitogenic effect was significantly greater only at the relatively high serum concentration of 20% (v/v), which is higher than serum concentrations routinely used in tissue culture. We have also shown that there is no clear relationship between the increased mitogenic effect of psoriatic serum and the clinical severity of psoriasis. We studied patients with psoriasis varying from a few small plaques to extensive skin disease. However, the 11 more stimulatory PHS samples were obtained from subjects with PASI scores of 4·5–51·0, demonstrating that a greater mitogenic effect was not consistent with more severe psoriasis. It was interesting to note that four of these 11 subjects had psoriatic arthropathy or Köbner reactions, while none of the other subjects had extracuticular features of psoriasis. This suggests that a serum factor may play a role in these particular manifestions of psoriasis. The majority of these 11 subjects were female (aged 18–74 years), but the significance of this finding is uncertain because our patient population included more females. Psoriasis is considered to affect males and females equally.

The second part of our work demonstrates that the sera taken at different times from the same psoriatic subject displayed different mitogenic effects, and this difference was not consistent with the corresponding change in skin disease. In two patients the growth stimulatory effects of the sera were marginally different, although the skin lesions had undergone marked resolution during the 5-month interval. With a third patient, there was a significant decrease in the mitogenic effect of her serum, despite a slight increase in skin disease and arthritis activity.

Our findings emphasize the apparent lack of association between the clinical severity of psoriasis and the increased growth stimulatory effect of psoriatic serum on cultured dermal fibroblasts. This, and the finding of a significantly greater mitogenic effect of psoriatic serum only at 20% concentration, suggests that a serum growth factor is unlikely to play a major role in the pathogenesis of psoriasis or in its humoral control.

However, in conclusion, we should caution that although the metabolism and proliferation of dermal fibroblasts is routinely studied in the presence of serum using cell culture,[3,4] it is uncertain whether these experimental findings can be correlated with the *in vivo* situation. *In vivo* fibroblasts obtain their cellular requirements from plasma, and not from serum; the difference being that serum contains a number of growth factors of platelet origin which participate in wound healing and inflammation.

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Aged versus young skin before and after transplantation onto nude mice

A.GILHAR, T.PILLAR AND M.DAVID

Skin Research Laboratory, Faculty of Medicine, Technion-Israel Institute of Technology, Haifa, Israel

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Summary The behaviour of aged skin transplanted onto nude mice was investigated to determine whether the skin maintains its histological features. Split-thickness skin grafts obtained from the unexposed skin on the thighs of healthy aged and young volunteers were grafted onto nude mice. A significant difference between the mean thickness of young versus aged epidermis was noted before transplantation ($P<0{\cdot}001$). The epidermis of aged and young skin showed an increase in thickness following engraftment with a mean increase in epidermal thickness of 18·8% in the young ($P<0{\cdot}01$) and 142·5% in aged skin ($P<0{\cdot}001$). The number of blood vessels in the aged skin was significantly lower than in the young skin, but a remarkable increase was found post-transplantation. These findings indicate that part of the typical histological changes of unexposed aged skin are reversible.

Aged skin is characterized by a variety of clinical and histological changes.[1] The histological changes in the epidermis are flattening of the dermo-epidermal junction, a decrease in height of the epidermis, occasional nuclear atypia, loss of melanocytes and fewer Langerhans cells.[1–5] Transplantation of human skin onto nude mice may provide a useful technique for studying the dependence of skin diseases upon the host.[6] Recently, we described the disappearance of the characteristic abnormalities of skin disorders such as alopecia areata,[7] lichen planus[8] and vitiligo[9] in skin grafts following transplantation onto nude mice.

The behaviour of aged skin transplanted onto nude mice has not previously been studied. We investigated whether aged skin maintains its histological features outside its old individual host.

Methods

Subjects

Eight healthy volunteers aged 73–89 years (mean age 81·7) and eight young healthy medical students, aged 23–28 years (mean age 25) were included in this study. After obtaining informed consent, split-thickness skin grafts were obtained using a hand-held keratotome from the unexposed areas of the thigh, after local anaesthesia with 1% lidocaine. The thickness of the grafts was 0·4 mm and their diameter was 1·2–1·5 cm.

Grafting and biopsy procedures

The methodology for transplanting skin grafts to the nude mouse has been described previously.[7–9] Four weeks after grafting, they were removed from each mouse.

Morphometric analysis

Histological assessment was performed by routine light microscopy of sections of grafts before and after skin transplantation. Measurement of the epidermal thickness and of different epidermal layers was carried out, in the vertical plane, with an ocular micrometer. Observations were taken at a minimum of 20 points along the epidermis and were selected to represent points of maximal and minimal thickness. The epidermal thickness represents the average distance from the stratum corneum to the tips of the dermal papillae and the rete pegs. The values for the epidermal thickness do not include the stratum corneum.

The number of blood vessels was measured in the whole area of the dermis of each specimen obtained from all patients. The results were given as mean number per mm^2. Dermal cells were enumerated without specific staining and were also given as mean number per mm^2.

Correspondence: Dr A.Gilhar, Laboratory of Skin Research, Technion Faculty of Medicine, P.O. Box 9649, Haifa 31096, Israel.

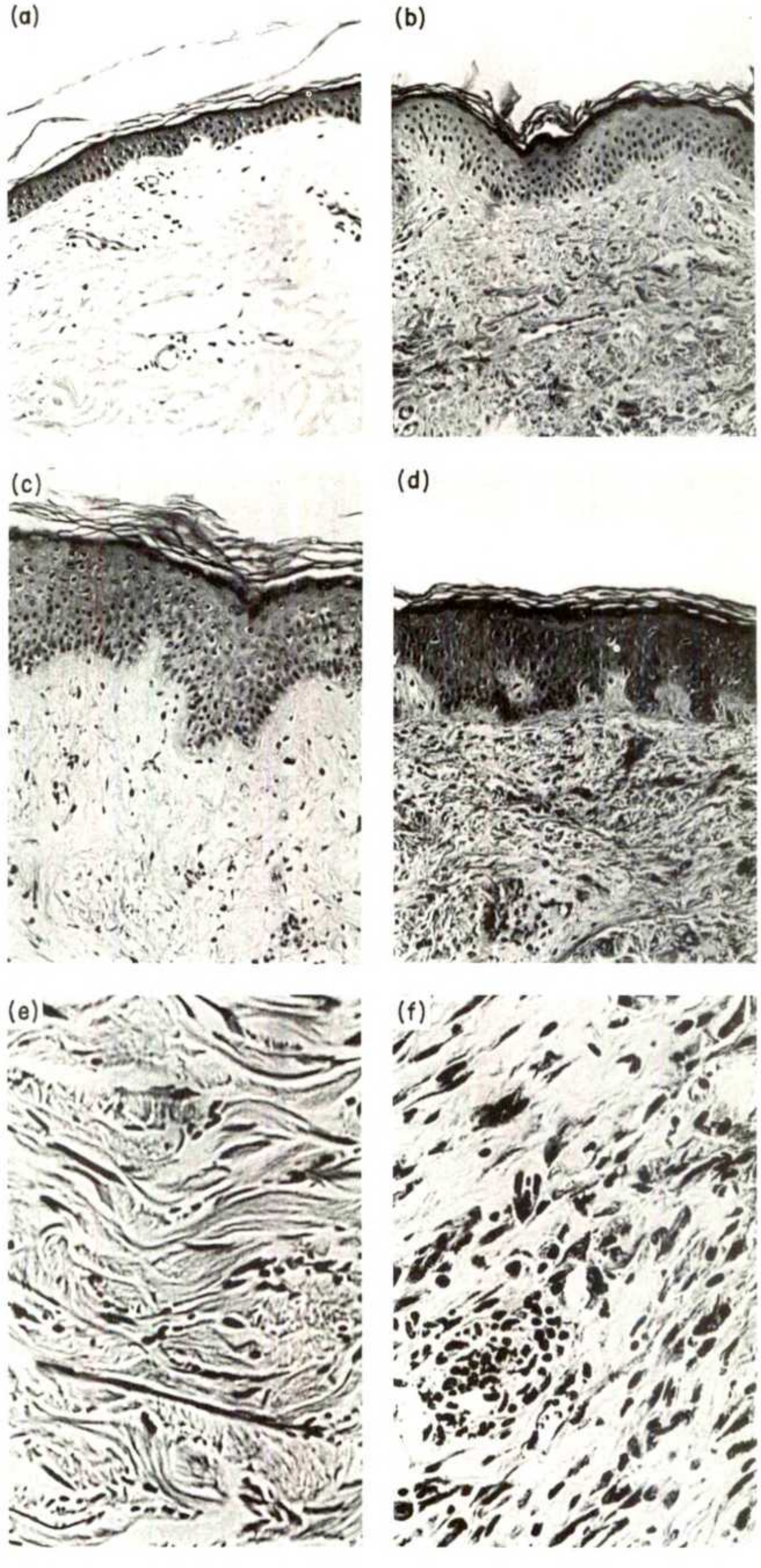

Figure 1. Epidermis obtained from (a) 76-year-old and (b) 24-year-old human skin. Transplantation of both (c) aged and (d) young skin onto nude mice caused increased thickness of the whole grafted epidermis. (e) The aged dermis was found to be relatively acellular and avascular. (f) After transplantation an increased number of dermal cells and blood vessels was found. (a, b, c and d × 150; e and f × 300).

Table 1. Aged versus young skin before and after skin transplantation

	Aged skin				Young skin			
	Before	After	%	$P<$	Before	After	%	$P<$
Epidermis (μm)*	62·3±12·6	151·1±27·7	142·5	0·001	111·9±5·8†	133±17·4	18·8	0·01
Stratum granulosum (μm)	8·8±1 8	16±5·3	81·8	0·01	11 8±1·2†	12·5±1·6	5·9	NS
Stratum corneum (μm)	25·6±6·6	49±8·5	91·4	0·001	26 1±10	42·3±9·5	62·0	0·01
Blood vessels/mm^2	1 3±0·64	10·1±6·1	676·0	0·01	5·2±1·2†	13·7±6	163·0	0·01
Dermal cells/mm^2	271·25±208	828·5±229·8	205·0	0·01	435±99	952±78·0	118·8	0·001

* Mean values (±SD) of epidermal thickness do not include stratum corneum.

† The difference in thickness of young and aged epidermis and stratum granulosum as well as the number of blood vessels before grafting reached statistical significance ($P<0·001$, $P<0·01$ and $P<0·001$, respectively).

Student's *t*-test for paired observations was used to compare the values of non-grafted skin with those of grafted skin.

Results

Morphometric analysis

A significant difference was found between the mean thickness of aged versus young epidermis (62·3±12·6 μm vs. 111·9±5·8 μm, $P<0·001$) (Table 1, Fig. 1a and b). Transplantation of human skin onto nude mice caused an increase in the thickness of the whole grafted epidermis (Fig. 1c and d). Morphometric analysis revealed an increase of 18·8% in the epidermal thickness of young skin (133±17·4 μm vs. 111·9±5·8 μm, $P<0·01$), which was even more marked in the stratum corneum (42·3±9·5 μm vs. 26·1±10 μm, $P<0·01$). No statistical difference was noted in the thickness of the stratum granulosum of young epidermis before and after transplantation of the skin onto the nude mice.

The increased thickness in the aged skin was more striking at 142·5% (151·1±27·7 μm vs. 62·3±12·6 μm, $P<0·001$), including the stratum granulosum (16±5·3 μm vs. 8·8±1·8 μm, $P<0·01$). The final thickness after skin transplantation was similar in both groups; the stratum corneum became significantly thicker after skin transplantation.

Dermal cells and blood vessels

The aged dermis was found to be relatively acellular and avascular (Fig. 1e). While a non-significant decrease in the number of dermal cells in the aged versus young skin was observed, following transplantation a statistically significant increase in number was noted in both aged (828·5±229·8/mm^2 vs. 271·25±208/mm^2, $P<0·01$) and young samples (952±78/mm^2 vs. 435±99/mm^2, $P<0·001$) (Table 1, Fig. 1f).

A significant reduction in the number of blood vessels in aged versus young skin was observed (1·3±0·64/mm^2 vs. 5·2±1·2/mm^2, $P<0·001$). After transplantation a significant increase was found in the number of blood vessels per mm^2 in both groups (1·3±0·64/mm^2 vs. 10·1±6·15/mm^2, $P<0·01$ in aged skin, and 5·2±1·2/mm^2 vs. 13·7±6/mm^2, $P<0·01$ in young skin).

Discussion

The present study confirmed previous observations of typical morphological changes of aged skin such as flattening of the dermo-epidermal junction,[10,11] a decrease in the vertical height of the epidermis[3] and a decreased number of blood vessels in the dermis.[3] We found a significant decrease in the vertical height of the epidermis of aged versus young skin ($P<0·001$), including the stratum granulosum ($P<0·01$). Previously,[9,12] an increase has been observed of 20–30% in the thickness of the epidermis of human split-thickness skin grafts from young donors after transplantation onto nude mice. The present study has confirmed this observation. Furthermore, we found a much greater increase in thickness in the aged skin of 142·5%. The final thickness of the epidermis after skin transplantation was, however, similar in both groups.

A marked reduction in the number of blood vessels in aged versus young dermis was noted, as has been described previously.[13] A significant increase in the number of blood vessels was observed in both groups after transplantation, which may be related to the process of graft acceptance. We have recently found a maximal vascularization in the edge of the graft[14] and some blood vessels in the grafted skin may be derived from the host. A striking hyperkeratosis was noted in both groups. Thickening of the stratum corneum after engraftment of human skin onto nude mice has been reported previously.[8,15]

The marked increase in epidermal thickness of aged epidermis after transplantation onto nude mice may be due to the elimination of age-associated factors which may slow down the proliferation rate of the epidermal cells. These factors may include an impairment of a variety of skin components such as a reduction of the blood supply and a loss of dermal volume.[1] The elimination of age-associated factors may lead to the enhancement and compensation of the epidermal turnover rate. An age-associated decrease in the epidermal turnover rate of approximately 50% between the third and seventh decades of life has been found.[16]

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Glomus tumours of the skin: an immunohistochemical investigation of the expression of marker proteins

W.M.HERBST, K.NAKAYAMA* AND O.P.HORNSTEIN

Department of Dermatology, University of Erlangen-Nürnberg, Erlangen, Germany

**Department of Dermatology, University of Occupational Environmental Health, Kitakyushu, Japan*

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Summary Immunohistochemical studies were carried out on cutaneous glomus tumours from 10 patients. The glomus areas in these tumours reacted strongly with anti-smooth muscle (CGA-7), anti-muscle (HHF-35) and anti-vimentin antibodies. Their neural supply was analysed using several nervous tissue markers (anti-neurone specific enolase, anti-S 100, anti-Leu 7, anti-neurofilaments). The results indicate a sprouting and proliferation rather than entrapment of pre-existing nerve fibres.

Glomus tumours (GT) are painful vascular lesions that originate from the arterial segment of the so-called Suquet–Hoyer canal, where a single layer of endothelial cells and four to six layers of glomus cells form a narrow lumen regulating the blood supply to the area.[1] Histologically there are two types, a solitary and a multiple type. Both are mainly composed of a compartment of modified epithelioid smooth muscle cells, which are often surrounded by undifferentiated cuboid cells. Whereas the solitary type of GT is an encapsulated proliferation of small blood vessels and epithelioid cells, the multiple type of GT has cavernous blood-filled spaces surrounded by the glomus cells. There is a variable surrounding stroma in these tumours.[1–3] The lesions are often painful, and this seems to depend on the presence of afferent nerve endings in the central portion and at the periphery of the tumours.

Immunohistochemistry offers the chance to analyse the expression of characteristic marker proteins by the use of mono- and polyclonal antibodies. On the other hand, the formation especially of specific anti-smooth muscle antibodies is difficult, as various muscle tissues such as skeletal, cardiac or vascular smooth muscle contain different actin iso-enzymes that vary in their amino acid sequences and isoelectric points.[4,5] These isoenzymes share more than 90% of the sequences of the entire molecule and a unique sequence exists only in the first 18 residues of the amino-terminus.[6–9] Consequently, analysis of the neural supply of GT by immunohistochemical investigation of the expression of nervous tissue markers has not been carried out up to now. Therefore, the aim of this study was to investigate the expression of both actin and nervous tissue markers in glomus tumours of both types to determine the histogenesis of the glomus cells and the origin of the innervation of these tumours.

Methods

Immunohistochemistry

Ten glomus tumours retrieved from the files of the Department of Dermatology, University of Erlangen–Nürnberg were studied. All had been fixed in 10% formaldehyde and embedded in paraffin wax. Immunohistochemistry using an indirect immunoperoxidase method was performed on 4–5-μm sections. Endogenous peroxidase activity in the sections was blocked by applying 3% H_2O_2 in methanol for 30 min. After washing the sections in phosphate-buffered saline at pH 7·2 a panel of monoclonal and polyclonal antibodies (Table 1) was applied overnight at 4°C. After washing three times with PBS, they were incubated with peroxidase-labelled anti-mouse antibody at 1:50 (Dakopatts, Denmark) for the monoclonal antibodies, or with peroxidase-labelled anti-rabbit antibody at a titre of 1:50 (Dakopatts, Denmark) for the polyclonal antibodies, for 30 min at room temperature and again rinsed with PBS. The specimens were then stained with 3-amino-9-ethylcarbimazole (Sigma Chemical Co., St Louis, MO, U.S.A.) and counterstained with haematoxylin and then mounted in glycerol gel (Dako, U.S.A.). Appropriate controls were carried out to test the specificity of the immunoreactions.

Correspondence: Dr W.M.Herbst, Department of Dermatology, University of Erlangen-Nurnberg, Hartmannstrasse 14, D-8520 Erlangen, Germany.

Table 1. Immunohistochemical reagents used in the study of glomus tumours

Reagent	Labelling	Source	Dilution
Anti-muscle* (HHF-35, 42 kDa)	Muscle cells	ENZO biochem‡	1.2000
Anti-smooth muscle* (CGA-7)	Smooth muscle cells	ENZO biochem	1:200
Anti-Factor VIII*	Endothelial cells	Dako§	1:100
Anti-vimentin* (V-9, 57 kDa)	Endothelial cells, smooth muscle cells	Dako	1:100
Anti-desmin† (PDE, 55 kDa)	Muscle cells	Euro Diag.¶	1:50
Anti-neurofilament* (NF, 200 kDa)	Neurones	Euro Diag.	1:50
Anti-Leu 7* (HNK-1, 110 kDa)	Schwann cells	Becton Dickinson**	1:100
Anti S-100 protein† (21 kDa)	Schwann cells	Dako	1:100
Anti-neurone specific enolase (NSE, 78 kDa)†	Neurones, sweat glands, melanocytes	Dako	1·250
Anti-synaptophysin*	Neurones, endocrine cells	Boehringer††	1:300

* monoclonal antibody.
† polyclonal antibody.
Dilution was chosen according to specific reaction combined with lowest background level examined on positive controls of normal human skin.
‡ ENZO biochem,
§ Dako, Hamburg, Germany.
‡ Euro Diag., Den Haag, The Netherlands.
** Becton Dickinson, Heidelberg, Germany.
†† Boehringer, Mannheim, Germany.

Table 2. Immunohistochemical labelling of glomus tumours of the solitary type

		Neural fibres of	
Reagent	Glomus cells	Central areas	Periphery
HHF-35	4/4	—	—
CGA-7	4/4	—	—
Factor VIII	—	—	—
Vim	4/4	—	—
Desmin	—	—	—
SYN	—	—	—
S-100	—	4/4	4/4
Leu 7	—	3/4	4/4
NF	—	2/4	3/4
NSE	—	3/4	4/4

—, non-immunoreactive in all cases.

Table 3. Immunohistochemical labelling of glomus tumours of the multiple type

		Neural fibres of	
Reagent	Glomus cells	Central areas	Periphery
HHF-35	6/6	—	—
CGA-7	6/6	—	—
Factor VIII	—	—	—
Vim	6/6	—	—
Desmin	—	—	—
SYN	—	—	—
S-100	—	5/6	6/6
Leu 7	—	3/6	6/6
NF	—	2/6	3/6
NSE	—	3/6	6/6

—, non-immunoreactive in all cases.

Results

On histology, the solitary type of glomus tumour (four cases) consisted of proliferating glomus cells arranged around vascular spaces and expanding into the adjacent connective tissue. The lumens of numerous small vessels were lined with a single layer of flattened, often elongated endothelial cells. A fibrous capsule surrounded the tumour and this contained a number of nerve fibres.

The multiple type of glomus tumour (six cases) showed wider vascular spaces than the solitary type and the endothelial cells again were surrounded by proliferating glomus cells. The fibrous capsule of the tumour was often missing and the nerve fibres sparse, in contrast to those found in the solitary type of GT. Close contact between small nerve fibres and the glomus cells was evident in the central parts of the tumour.

The immunohistochemical labelling patterns of the different antibodies for both types of GT are shown in Tables 2 and 3. The glomus cells of both types generally showed cytoplasmic staining to a varying degree with anti-HHF-35 (Fig. 1) and anti-CGA-7 (Fig. 2) and anti-

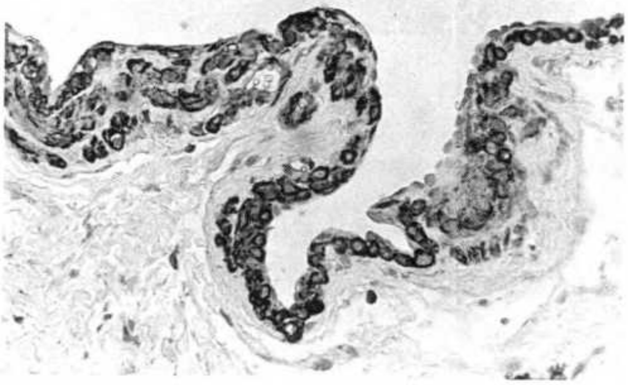

Figure 1. Multiple type of glomus tumour showing strong cytoplasmic anti-muscle antibody (HHF-35) immunoreactivity in the glomus cells surrounding endothelial-lined vessel lumen (× 142).

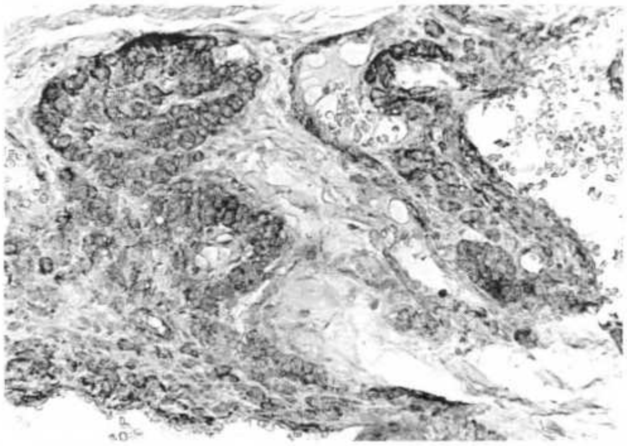

Figure 2. Moderate to strong anti-smooth muscle antibody (CGA-7) immunoreactivity in the cytoplasm of glomus cells (× 142).

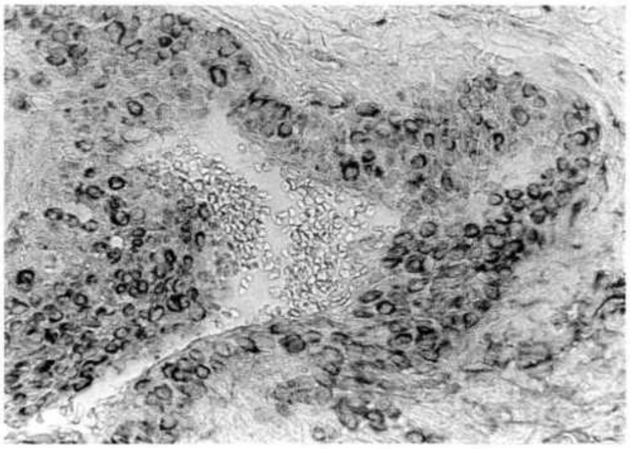

Figure 3. Moderate to strong anti-vimentin (VIM) immunoreactivity in the cytoplasm of both glomus and endothelial cells. Glomus tumour of the multiple type (× 142).

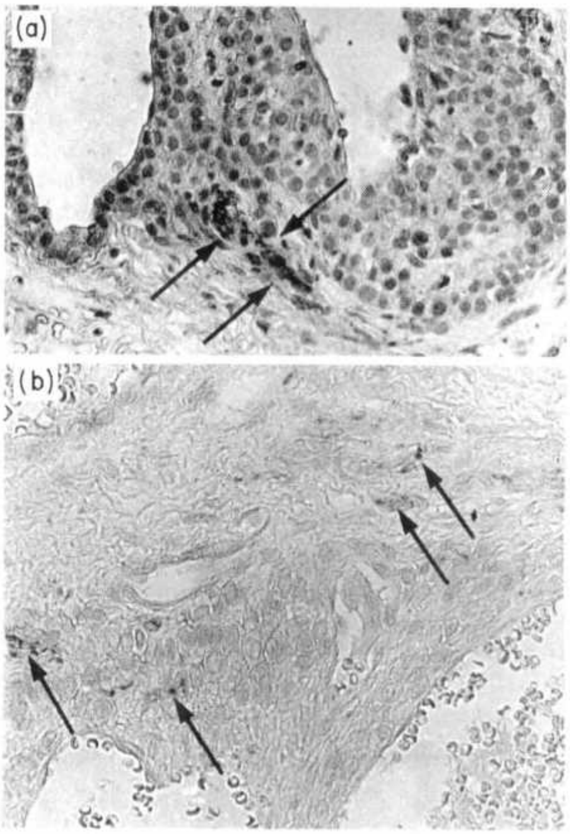

Figure 4. Anti-NSE immunoreactivity (a) along the periphery of a glomus tumour and (b) within the stroma of the tumour (× 200).

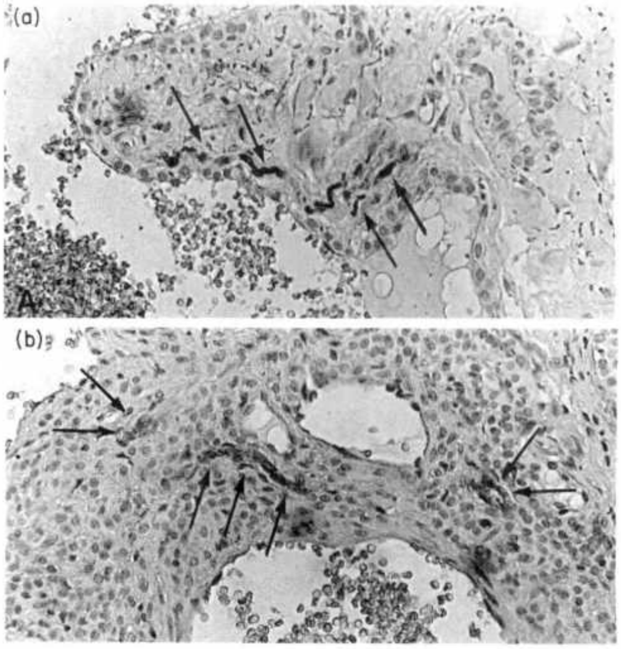

Figure 5. (a) Strong anti-S-100 protein immunoreactivity in the central part of the stroma of a glomus tumour of the solitary type (× 177) and (b) along a blood-filled space at the periphery of a glomus tumour of the multiple type (× 142).

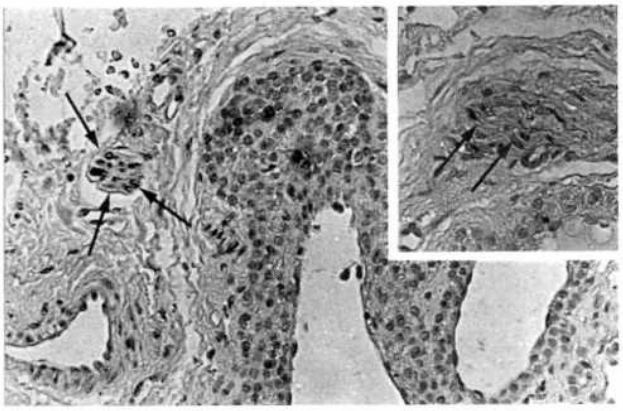

Figure 6. Anti-Leu 7 immunoreactivity (arrows) and anti-NF immunoreactivity (inset, arrows) located at the periphery of the glomus tumour (× 142, inset × 178).

vimentin antibody (Fig. 3), which also labelled endothelial cells. Desmin immunoreactivity was not expressed by the glomus cells. In both types of GT most of the nerve fibres were located at the periphery of the tumours showing neurone-specific enolase immunoreactivity (NSE) as shown in Figure 4. S-100 (Fig. 5) and neurofilament immunoreactivity was exhibited both in the central and peripheral parts and Leu 7 reactivity was observed predominantly in the periphery of the tumour (Fig. 6).

Discussion

The origin of glomus tumours was uncertain for a long time, but it was then recognized using electron microscopy that smooth muscle cells are the major component of both types of tumours.[3] Highly differentiated smooth muscle cells were identified that contain in their cytoplasm many myofilaments and associated dense bodies.[3] In our study we identified vimentin with an antibody to intermediate filament proteins,[10] but no desmin immunoreactivity was found in the cytoplasm of the glomus cells. This corresponds to normal vascular smooth muscle cells.[11] Some authors have correlated desmin-positive and desmin-negative vascular smooth muscle cells with different functional states,[12,13] whereas others have postulated that the expression of desmin may vary between the different parts of the vascular tree.[10] The demonstration of desmin/vimentin immunoreactivity, therefore, does not seem to be of value for the diagnostic differentiation of vascular disorders.

Several polyclonal anti-actin specific antibodies have been developed[7–9] and a monoclonal anti-actin antibody (HHF-35) which reacts with both smooth and striated muscle actins and which binds to smooth muscle, skeletal muscle and cardiac muscle actin[7] has recently been reported.[8] Another smooth muscle-specific antibody (CGA-7) that reacts neither with endothelial cells nor with fibroblasts was recently used for the identification of myogenic tumours of smooth muscle origin such as leiomyomas and highly differentiated leiomyosarcomas.[9] Both antibodies seem to react with different epitopes of the same actin molecule.[9] The immunohistochemical demonstration of both muscle- and smooth muscle-specific anti-actin immunoreactivity inside the glomus cells seems to confirm the hypothesis that these tumours develop out of the arterial segment of the Suquet–Hoyer canal. Nevertheless, these findings do not indicate whether the glomus cells are specialized endothelial cells or genuine smooth muscle cells.

Immunolabelling for Factor VIII-related antigen has provided further proof for the smooth muscle nature of the glomus tumours by labelling the endothelial and not the glomus cells. In contrast to the antibodies binding to nervous tissue proteins the anti-muscle antibodies employed in this study showed relatively constant cytoplasmic immunoreactivity for both types of GT.

The S-100 protein that is predominantly localized in glial cells of the brain has considerable importance as a neurogenic tumour marker for diagnostic histopathology.[14] In both types of GT there is positive labelling for S-100 protein in the nerve fascicles at the periphery of the tumours. This may be due to the presence of myelinated fibres in this region. In central areas there was no expression of myelin basic protein, a component of the myelinic nerve sheath.[15] Another antibody, Leu 7, that has been shown to react with myelin-associated glycoprotein in neuroectodermal tissue,[16] showed positive labelling that was predominantly at the periphery of the tumours. The occurrence of Leu 7 and S-100 immunoreactivity in a few of the lesions could be due to unfolding of peripheral parts of the tumour. However, both S-100 and Leu 7 immunoreactivity are detectable in proliferating and normal Schwann cells and these cells may be present.

The NF immunoreactivity may reflect the presence of entrapped nerve fibres or those that have undergone regenerative sprouting. The parallel non-expression of Leu 7 immunoreactivity in the glomus cell tumour compartment indicates an origin from sprouting and proliferating Schwann cells rather than the entrapment of pre-existing nerve fibres. These conclusions are supported by a previous study which found no myelin basic protein inside the glomus tumours.[15]

The antibody neurone-specific enolase (NSE) is directed against the glycolytic enzyme enolase and is located both in the cells of the brain and in peripheral neurones.[17] It is expressed in neuronal, glial and neuroendocrine cells and also a host of unrelated cells. Experimental studies with chickens have revealed evidence of relatively late production of NSE in the development of neurones.[18] There appear to be no significant differences in the expression of NSE in the two types of GT. At the ultrastructural level, the multiple type of GT shows less neural structures than seen in the solitary types.[3,19] As previously described,[19,20] there was close contact of some nerve fibres with glomus cells as was also observed in our study.

Synaptophysin[21] was not found, but this is not surprising as the tissue examined was formalin-fixed rather than frozen specimens and had not been treated with trypsin or other proteolytic agents which may lead to false positive reactions.[21,22]

The pain in glomus tumours is considered to be transmitted via the sensory nerve endings both within the tumours and at their periphery.[15] Substance P (SP) and calcitonin gene-related peptide are two peptides associated with sensory fibres and the afferent innervation of vessels.[23,24] These peptides mediate vasodilatation via the antidromic stimulation of nerve fibres[25] and are generally accepted as potent vasodilators of the microvasculature.[26,27] SP-immunoreactive nerve fibres have been reported in the parenchyma of solitary type GT, but not around endothelial cells[15] where we were able to demonstrate the presence of nerve fibres. A study comparing afferent nerve fibres in the solitary type of GT, which are known to be often painful, with the multiple type of GT has as yet not been carried out.

The application of specific anti-actin antibodies has demonstrated the origin of GT to be specialized smooth muscle cells derived from the vessel walls. The analysis of the expression of nervous tissue markers appears to indicate a sprouting and proliferation of Schwann cells rather than an entrapment of pre-existing nerve fibres.

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Lower leg subcutaneous blood flow during walking and passive dependency in chronic venous insufficiency

K.PETERS, J.H.SINDRUP, L.J.PETERSEN, F.OLIVARIUS, L.DANIELSEN AND J.K.KRISTENSEN

Department of Dermatology, University of Copenhagen, Bispebjerg Hospital, Copenhagen, Denmark
Bispebjerg Hospital, Copenhagen, Denmark

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Summary

The blood flow in the subcutaneous adipose tissue of the lower leg of eight normal subjects and 19 patients with chronic venous insufficiency was measured. The ^{133}Xe-washout technique was used with portable CdT1(C1) detectors and a data storage unit. Only those patients with ulcers and a systolic blood pressure at the toe of ≥ 60 mm were investigated.

In the controls the relative blood flow during sitting was 0·61 (range 0·35–0·80). In the patients it was 0·46 (range 0·22–0·87). This difference was not significant. During walking the blood flow increased in controls as well as in the patients compared to the value determined in the sitting position ($P = 0{\cdot}0078$ and $P = 0{\cdot}0028$, respectively, Wilcoxon matched-pairs test). The relative blood-flow rate during walking was 0·96 (range 0·60–1·58) in the controls, and 1·04 (range 0·49–1·46) in the patients. The difference between the normal subjects and the patients was not significant ($P = 0{\cdot}79$).

We conclude from our studies that patients with venous insufficiency are able to increase their blood flow during walking to the same extent as normal controls.

Lower leg venous ulceration appears to be due to an elevated venous pressure in the legs during upright posture and exercise.[1] This venous hypertension causes stretching of the endothelial pores[2] with structural changes in the capillary bed.[3,4] The delivery of oxygen to the skin is impaired, contributing to the cutaneous changes seen. The sustained high pressure in the veins of the leg caused by the destruction of the valves and incompetent perforating veins results in pain and swelling of the leg, with eczema, fibrosis, pigmentation and ulceration of the skin. Patients with incompetent perforating veins are unable to reduce the pressure in the veins of the foot during exercise to the same degree as seen in normal subjects.[5] There is evidence that venous distension, corresponding to an increase in venous transmural pressure of 25 mmHg or more, elicits an arteriolar constriction in the skin, subcutaneous fat and in skeletal muscle by a local sympathetic axon reflex mechanism (the veno-arteriolar reflex).[6] Thus in the upright position the local veno-arteriolar reflex, as well as the central baroreflex impulses contribute to the vasoconstriction. Both reflexes induce a constriction of the same magnitude, but the effect is not additive.[7]

In normal subjects activation of the vein pump during passive lowering of the leg decreases venous transmural pressure. The local veno-arteriolar reflex is thereby abolished and there is an increase in the subcutaneous blood flow. Contrary to this, patients with chronic venous insufficiency have a continuing activation of the local reflex during raising of the heel.[8] The transcutaneous O_2 tension was measured during standing and after exercise[9] and following activation of the vein pump, O_2 tension increased in normal subjects, but remained low in patients with venous insufficiency. This reaction could be restored to normal following the surgical correction of their venous insufficiency.[10] These studies however have been criticized on the grounds that the workers used an unheated O_2 sensor at 37°C.[11] It has been shown that it is necessary to use a heated sensor at 44°C to produce consistent results with this method.[12] The results of Dodd *et al.* have not been confirmed.[13]

The present study was carried out to investigate the regulation of subcutaneous blood flow in patients with chronic venous insufficiency. A recently developed method for measuring blood flow in subcutaneous tissue based on the ^{133}Xenon-washout technique was used.

Methods

Nineteen patients with phlebographic evidence of

Correspondence: Dr J.K.Kristensen, Department of Dermatology, Bispebjerg Hospital, DK-2400 Copenhagen NV, Denmark.

incompetent perforating veins and an age range of 43–89 years were investigated and compared with eight normal age-matched controls aged 40–74 years. Only the patients with ulcerated legs were investigated and six of these had bilateral ulcers. In all patients the distal blood pressure in the legs was measured using the strain-gauge technique. All had systolic toe blood pressure of $\geqslant 60$ mmHg. This level has been found to be of value for prognosis and to exclude cases where atherosclerosis was a major cause of ulceration.[14] None of the subjects investigated were suffering from diabetes.

Measurements

The blood flow on the lateral aspect of the lower leg 10 cm proximal to the level of the malleolus or just proximal to the ulcerated area was measured. Saline solution (1 ml) of the gas ^{133}Xe (5 mCi) was injected into a gas-tight (Mylar membrane) chamber that was attached to the skin surface by means of double-sided adhesive tape. The exposure time was 60–120 s, during which time there was diffusion of the tracer through the epidermis and into the dermis and subcutaneous tissue. This solution was withdrawn back into the syringe and the surplus of ^{133}Xe was removed. The diameter of the labelled skin was 4 cm. This labelling technique has been previously described.[15,16] After 1 h a portable CdT1(C1) semi-conductor detector was mounted over the central portion of the labelled area and fixed to the skin surface by means of adhesive tape that has been found to have no influence on the measurements of ^{133}Xe.[16] A single layer of Mylar membrane was interposed between the detector and the skin surface to avoid accumulation of ^{133}Xe during measurement in the rubber cap of the detector. The measurements were not commenced until 90 min after the labelling procedure to allow the lipophilic tracer to be deposited in the subcutaneous fat.[15]

The activity in the subcutaneous depot of ^{133}Xe was recorded continuously by a Memolog 500 or 600 system (B.Simonsen Medical A/S, Randers, Denmark).[17] During the measurements the counts were accumulated in 30-s intervals in a portable data storage unit. Washout was recorded with patients supine (reference 1), sitting with passive dependent legs (test 1), supine (reference 2), during walking (test 2) and finally supine (reference 3). Each period of test measurement lasted 15 min while the reference values were recorded over 10 min.

Calculation and statistics

The collected counts were computed and the logarithmically transformed clearance curves for the reference and test periods were plotted.

The subcutaneous blood flow (SBF) can be calculated from

$$\mathrm{SBF} = \lambda \times \mathrm{k} \times 100\ \mathrm{ml} \times (\mathrm{min} \times 100\ \mathrm{g})^{-1}. \qquad (1)$$

Lambda ($\lambda = 10\ \mathrm{ml} \times \mathrm{g}^{-1}$ in subcutaneous tissue) denotes the tissue to blood partition coefficient of the tracer in the tissue investigated. Assuming λ to be constant throughout the measurements, relative changes in the washout rate constant (k) reflect relative changes in the SBF.

The measurements were divided into five phases as described above. Relative blood flow during sitting (RSBF(S)) was calculated as:

$$\mathrm{RSBF(S)} = \frac{\mathrm{k\ (test\ 1)}}{(\mathrm{k\ ref\ 1} + \mathrm{k\ ref\ 2}) \times 0{\cdot}5}. \qquad (2)$$

Relative blood flow during walking (RSBF(W)) was calculated as:

$$\mathrm{RSBF(W)} = \frac{\mathrm{k\ (test\ 2)}}{(\mathrm{k\ ref\ 2} + \mathrm{k\ ref\ 3}) \times 0{\cdot}5}. \qquad (3)$$

In controls and patients with bilateral ulcers the mean of the results in each leg was used for further calculation. Differences between the controls and the patients were compared by the non-parametric Mann–Whitney test while differences in groups during movement were compared by the non-parametric Wilcoxon matched-pairs test or the Page test for trend.

Results

An example of the washout curves obtained is shown in Figure 1. The results are presented in Table 1. During sitting, blood flow decreased significantly in the controls as well as in the patients. RSBF(S) was found to be 61% and 46% in the controls and the patients, respectively. During walking the median blood flow was unchanged compared to the supine position in the controls as well as in the patients. The difference between sitting and walking was significant in the controls ($P = 0{\cdot}0078$) and in the patients ($P = 0{\cdot}0028$) using the Wilcoxon matched-pairs test.

The relative blood flow did not differ significantly between patients and controls either during sitting ($P = 0{\cdot}33$) or during walking ($P = 0{\cdot}79$). The resting supine washout constant tended to be more variable in

Table 1. Blood flow in subcutaneous tissue

	Controls ($n=8$)	Patients ($n=19$)
(k) min^{-1}	0·0045 (0·0033–0·0055)	0 0055 (0·0032–0·0179)
RSBF(S)	0·61 (0·35–0 80)	0·46 (0·22–0·87)
RSBF (W)	0·96 (0·60–1·58)	1 04 (0·49–1·46)

All values are medians and 95% confidence limits. (k) is the resting supine washout rate constant. RSBF(S) and RSBF(W) are relative subcutaneous blood flow during sitting and walking, respectively.

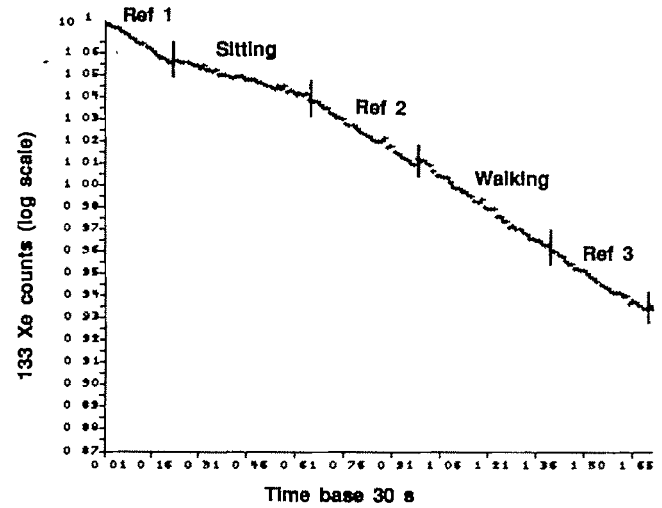

Figure 1. A representative washout curve from a patient with a leg ulcer showing the curve segments used in the calculation of washout rates and relative blood flow.

the patients but the difference was not significant ($P=0{\cdot}31$).

A paired statistical comparison of reference 1 and reference 3 using the Wilcoxon test gave $P=0{\cdot}35$ (two sided), indicating that the reference values were reproducible. A Page test for trend applied on the three reference values showed $P=0{\cdot}51$ (one sided), confirming reproducibility.

Discussion

The method employed in this study did not permit quantitative measurements of subcutaneous blood flow in the legs. Only relative changes during the different manoeuvres were recorded.

A normal reaction to orthostatic stress while standing was observed in our study in patients suffering from chronic venous insufficiency. Upright posture is associated with a fall in the aortic pulse pressure which in humans triggers a strong arterial baroreflex.[18] A fall in the central venous pressure is also observed which causes vasoconstriction but may also augment the reflex effects of reduced arterial pulse pressure. In addition a local sympathetic axon reflex[6] appears to be triggered by venous congestion. The receptor sites appear to be in small veins in the skin, muscle, subcutaneous fat and possibly other tissues as well.[6] The effector sites are in the arterioles supplying these tissues. This axon reflex may be an important adjunct to centrally mediated sympathetic nervous activity during the upright posture.[7]

During walking, the blood flow was increased in the controls as well as in the patients compared to that while standing and reached the level observed during rest in the supine position. To our knowledge, the subcutaneous blood flow in the lower leg during walking has not been previously measured, but the results compared favourably with those found in the subcutaneous tissue of the thigh and abdomen during bicycle exercise.[19] The 99-m Tc-pertechnetate clearance from the leg or abdomen was found to be unchanged during exercise, indicating that the subcutaneous blood flow rate was unaltered.

In the upright posture 70% of the total blood volume is found below the heart, and 75% of this volume is found in distensible veins. Once the veins fill and their valves open, they are subjected to the full hydrostatic pressure of a continuous column of blood between them and the heart. In this position, before exercise begins, vasoconstriction and not venoconstriction appears to reduce the rate at which dependent veins fill. The veins do eventually fill to a volume that depends on the hydrostatic force, their compliance, and the degree to which their transmural pressure is reduced by resting tension in adjacent muscles.

At the onset of exercise, muscle contractions break up the volume of blood in the veins of the legs and blood is pumped to the thorax, restoring central venous pressure, central blood volume and the stroke volume of the heart to the values observed during rest in the supine position. Therefore it may not be surprising that subcutaneous blood flow in normal legs is found to be unchanged compared to the resting situation.

In the patients the finding of a normal blood flow during walking was not in accordance with the expected maintained high venous transmural pressure during exercise. Sympathetic vasoconstrictor outflow to most major organ systems increases in proportion to the severity of exercise.[20] Cutaneous, and probably subcutaneous, vessels constrict at the onset of exercise but the fall in skin blood flow is only transitory.[21] Once skin temperature starts to rise, vasodilator outflow to the skin masks the effects of any maintained vasoconstrictor activity. Our results show that moderate exercise abolished the vasoconstrictor response to the increase in venous transmural pressure in the lower legs in the controls as well as in the patients. The mechanisms whereby this vasoconstriction is overcome might not be the same in these groups. The effect of exercise might be due to one or more of the following possibilities. Activation of the vein pump may prevent the increase in venous transmural pressure in the dependent leg. Exercise might cause a change in metabolism and liberation of metabolites in subcutaneous tissue affecting the reactivity of vascular smooth muscle cells, providing the exercise is prolonged. It has been shown that the adipose tissue blood flow rate increases during prolonged exercise and that this increase seems to be secondary to metabolic events connected to lipolysis.[22] This factor might not be relevant during the brief period of exercise as used in the present study. Increased skin temperature during exercise might mask any maintained vasoconstrictor activity in cutaneous vessels due to increased vasodilator outflow to the skin and subcutaneous tissue.

In conclusion, the sustained increase in venous pressure observed in patients with chronic venous insufficiency did not affect the subcutaneous blood flow in the lower legs during walking. This does not imply that a decreased oxygen extraction due to diffusion blocks, elongated tortuous capillaries and increased diffusion distances might not be important in the development of venous ulcers. The importance of these factors needs to be determined in future studies.

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Photoelectric quantitative evaluation of argon laser treatment of port wine stains

R.A.NEUMANN, R.M.KNOBLER AND A.P.LINDMAIER
Department of Dermatology II, University of Vienna, Alserstrasse 4, 1090 Vienna, Austria

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Summary

Sixty patients with port wine stains were treated with the argon laser and graded visually into four subjective categories for changes in skin colour. For objective quantitative evaluation a photoelectric colorimeter was used. Reproducible definition of the lightness of the lesions into numbers was obtained. Differentials in lightness between normal contralateral skin and the portwine stain before and after treatment were quantified for each individual. The objective evaluation revealed a statistically significant difference between the four categories ($P<0{\cdot}05$). The best clinical therapeutic effect was found to correlate with only 80–90% blanching. The average lightening obtained was 46·5%. Local heating of portwine stains in 12 patients showed no measurable darkening in the colour of the lesions, whereas the normal contralateral skin reacted with marked reddening. Determination of changes in lightness by photoelectric colorimetry provides an objective, quantitative means to evaluate the effects of laser treatment of port wine stains.

Port wine stains (PWS) are congenital vascular naevi, characterized by a wide variation in size, localization, and colour. On histology there are numerous ectatic vessels in the upper and mid-dermis. Several treatment modalities such as X-ray, cryotherapy with liquid nitrogen or dry ice, and surgical excision have been employed, with little or no success. However, since the introduction of laser therapy[1–3] the prognosis of these lesions has markedly improved. The most disfiguring aspect of PWS is the colour of the lesions.

In dermatology the description of the colour of a lesion is of great importance, however, colour is a matter of relative subjective perception and definition. In PWS, a quantitative determination of the colour would be of great value for an objective assessment of the results of laser therapy. Usually, this is through subjective observation, using either graded colour charts[4,5] or 'before and after' photographs.[6–8] Up to the present time there have been relatively few attempts at an objective evaluation.[9–13]

In the present study 60 patients suffering from PWS were treated using an argon laser. Evaluation of treatment results was done by visual grading based on comparative photographs and by means of a portable hand-held photoelectric colorimeter, which allows an objective non-invasive method for quantitative evaluation of changes in lightness of the skin. Changes of PWS and normal skin in response to local application of heat were also measured.

Methods

Patients

Sixty patients (37 females, 23 males) suffering from PWS were treated with the argon laser. Their age range was 11–67 years (mean 24·3 years). The most common location was the head and neck, followed by the trunk and extremities. The colour varied from deep purple to pink and there was no specific age clustering. In four patients the original smooth surface of the PWS became more nodular but this only occurred in facial lesions.

Treatment

An argon laser type Coherent 920 (Coherent Medical Group, Palo Alto, CA, U.S.A.) was used. A pulse duration of 0·2 and 0·3 s, a beam diameter ranging from 1 to 2 mm, and a power setting of 1·5–3·0 W were selected in order to produce clear white spots with no initial grey or black colouration of the overlying epidermis. A constant distance of 2 cm from handpiece to tissue was kept by a spacer. The power was measured using a Laser Power Meter 210 of the Coherent Medical Group. All patients

Correspondence: Dr R.A.Neumann.

were treated using the separate spot technique, preserving about 0·5–1 mm untreated skin between the spots. Post-operatively, betamethasone in combination with gentamycin was applied twice daily for 8–10 days. In our experience a minimum interval of 4 months between treatments is required. The final evaluation was made 6 months after the last treatment.

Photographs were taken before and after treatment with specific attention to uniformity of pose and lighting.

Photoelectric colorimetry

In addition to visual grading, patients were evaluated quantitatively by means of a portable hand-held colour analyser: the Chroma Meter II Reflectance (CMR) (Minolta Camera Co. Ltd., Osaka, Japan). The system consisted of an optical head connected by an optical-fibre cable with a control console. It measured the reflectance ratio between the light emitted by the optical head and the light reflected from the subject. The measuring head contained a high-power pulsed xenon arc (PXA) lamp which provided diffuse illumination from a controlled angle for vertical viewing and constant, even lighting on the subject. Six high-sensitivity silicon photo cells filtered to detect primary stimulus values for blue, green, and red light, were used. The spectral response closely approximated the colorimetric Standard Observer curves of the Commission Internationale de l'Eclairage (CIE). The double-beam feedback system detected any slight deviation in the xenon light's spectral distribution and a microcomputer compensated for them. Readings were processed by the built-in computer and were presented as digit numbers on the liquid-crystal display. The measuring area at the tip of the measuring head was only 8 mm in diameter, allowing numerical readings of very small samples without cutoff.

The CMR converts colours into a common numerical code, where L* means lightness, its value ranging from zero (black) to 100 (white). The colour hue is represented by a*, indicating the red–green axis, and by b*, indicating the yellow–blue axis.

Improvement of a PWS always means a lightening towards normal skin colour. Therefore we tried to establish whether the blanching effect of PWS due to laser treatment could be described sufficiently by changes in lightness, L*.

Measurements

Before use the colour analyser was calibrated by means of the Standard-White Reflector Plate (Minolta Camera Co. Ltd., Osaka, Japan). Measurements were performed under standardized conditions in all patients. The patients had to rest for 20 min in a temperature-controlled room (20°C) before measurements were made and they were instructed not to use any cosmetics or other local substances or new medication for at least 24 h prior to examination.

The measuring head was held perpendicular to the skin surface and the aperture was fitted with an applicator, to avoid compression of subcutaneous capillaries. After pressing the measuring button the obtained readings appeared within a few seconds on the display. All the measurements were performed in triplicate and mean values were used. This method was highly reproducible.

Before the start of laser treatment a representative area of the PWS and then the equivalent contralateral uninvolved area was measured. In 11 patients presenting with non-homogeneous lesions the darkest area was measured. For the final evaluation of treatment outcome the treated area and the contralateral normal skin were measured at least 6 months after the last treatment. If the two measurements of the normal skin differed, the measurement of the treated lesional skin was increased or decreased by the same value.

The effect of local heat on 12 patients was measured as previously described.[12] A glass container with a 2-cm-diameter base, full of water at 43°C, was applied to the skin for 2 min. First the PWS and then the contralateral normal skin were tested and evaluated visually and by photoelectric colorimetry.

Statistics

The statistical evaluation of the data was performed by the modified Mann–Whitney test for multiple non-parametric comparisons and according to the Wilcoxon two-sample test. A P value of less than 0·05 was considered significant.

Results

Subjective evaluation

Evaluation of the response to treatment was made visually by the therapist based on photographic documentation and was graded into four categories: excellent result with nearly total blanching, good result with marked lightening, fair result with only moderate lightening, and poor result with no response. Eight patients (13·3%) were judged as having excellent, 24

Table 1. Objective classification of treatment results by photoelectric colorimetry

Blanching	Patients	
(%)	(*n*)	(%)
80–90	5	8·3
70–80	3	5·0
60–70	8	13·3
50–60	10	16·7
40–50	10	16·7
30–40	13	21·7
20–30	5	8·3
10–20	4	6·7
0–10	2	3·3
Total	60	100·0

patients (40%) good, 22 patients (36·7%) fair, and six patients (10%) had a poor response to treatment.

The classification of the individual using this subjective method was clear in the excellent and poor responders, but difficult in the good and fair responders. In a retrospective evaluation of all patients the classification was easier.

Objective evaluation

For the evaluation of treatment the difference between the values for lightness L* of normal skin and non-treated PWS skin was considered as 100%. Blanching was assessed as the per cent change in lesional values for lightness L* after treatment.

The values for lightness L* of PWS were in the range 39·1–61·0 units (median 49·8), and of normal contralateral skin 50·4–70·4 units (median 61·0). The difference between these two values before treatment was in the range 3·2–20·0 units (median 10·95), and after treatment ranged from 1·2 to 13·0 units (median 5·15). A good correlation could be established between these objective measurements of PWS lightness and the subjective grading of colour.

The results of this objective evaluation showed that none of our patients had a 100% blanching (Table 1). An excellent and a good response to treatment are shown in Figures 1 and 2, respectively (Table 2). In the patient with the subjectively assessed excellent result there was a lightness difference of 10·3 units between the PWS and normal contralateral skin before laser treatment. The increase in lightness resulting from treatment was by 9·2 units, which corresponded to a blanching of 89·3%. In the patient with a good result the increase in lightness towards that of normal skin was 6·4 units or 51·2%, leaving a difference of 6·1 units after treatment from normal skin lightness.

The results of CMR measurements were compared with those obtained by subjective visual grading (Fig. 3). We found significant differences ($P<0{\cdot}05$) between nearly all distribution patterns of objective measurements in the four categories of subjective treatment

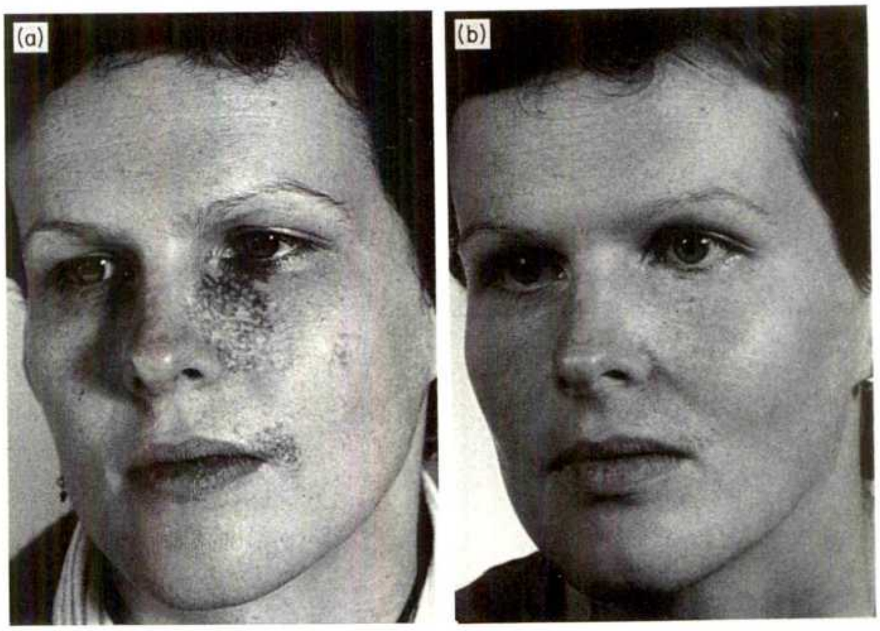

Figure 1. (a) Port wine stain before laser treatment; (b) excellent result after treatment corresponding to 89·3% blanching, evaluated by photoelectric colorimetry.

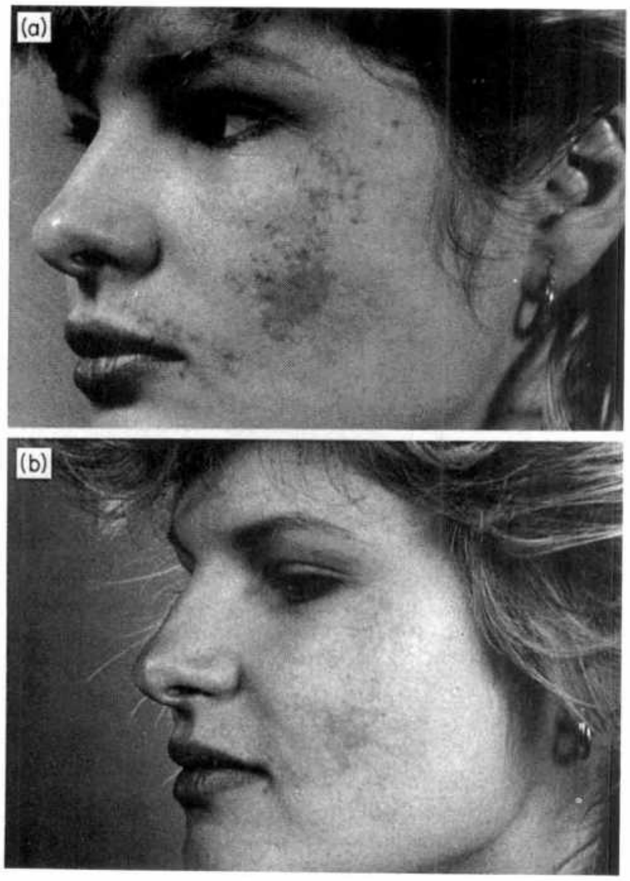

Figure 2. (a) Port wine stain on left cheek before laser treatment; (b) good result after treatment with marked blanching corresponding to 51·2% increase in lightness measured by photoelectric colorimetry.

Table 2. Representative samples for an excellent and a good treatment result assessed by photoelectric colorimetry

	Units for lightness L*			
Subjective result	PWS before treatment	PWS after treatment	Normal contralateral skin	Blanching (%)
Excellent	54·0	63·2	64·3	89·3
Good	50·3	56·7	62·8	51·2

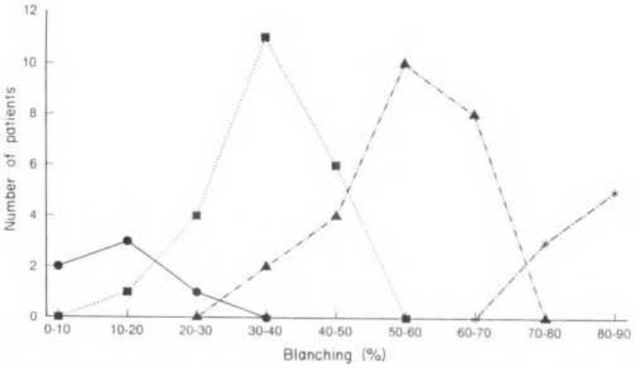

Figure 3. Subjective evaluation (*, excellent; ▲, good; ■, fair; ●, poor) of effect of argon laser treatment on PWS correlated with photoelectric assessment (% blanching).

Table 3. Subjective improvement categories as determined by objective assessment of blanching

		Blanching (%) assessed by photoelectric colorimetry			
Subjective evaluation	Patients (*n*)	Median	Q3–Q1*	Minimum value	Maximum value
Excellent	8	81·87	5.03	73·75	89·32
Good	24	55·06	8·19	37·50	68·30
Fair	22	34·90	7·57	19·69	46·95
Poor	6	13·96	8·65	2·05	26·92

*Q3–Q1 denotes the difference between the upper and the lower quartile.

evaluation (Table 3). When comparing the categories fair and poor, due to the small number of observations in the poor category, no significant differences could be noted.

We further divided all patients according to the value of the differential of lightness between PWS and normal skin into two groups of 30 patients. The relative median improvement in the first group, with less differential in lightness, was 47·38% (range 8–89%), and in the second group it was 45·52% (range 2–84%). Thus, the remaining difference in lightness between normal skin and treated PWS showed a highly significant difference ($P<0·001$) between the two groups: group 1, median 4·35 units (range 1·1–7·8 units); group 2, median 7·15 units (range 1·8–16·3 units).

Local heating

All PWS examined showed no visible response to local application of heat. With the CMR no differences between the measurements before and after heat application were found.

In contrast, on uninvolved contralateral skin local heating led to a visible reddening of the skin due to vasodilation. This increase in the red colour tone corresponded to a decrease in lightness L* in the range of 2·3–6·4 units (median 3·1 units).

Discussion

Until now, the results of laser treatment of port wine stains (PWS) have mostly been assessed by subjective direct observation and comparative photographs[4–8]. These methods are dependent on the individual's interpretation of colour. Although histological analysis of treated areas provides an objective means for evaluating treatment response,[14,15] its value is limited because it is invasive, not necessarily representative for the entire lesion, and often suggests results that do not correlate with the clinical appearance.

Photoelectric colorimetry (PC) measures the reflectance through different filters in comparison to standard surfaces. Hitherto this method has provided useful data regarding the quantitation of erythema and tanning responses to ultraviolet radiation[16–19] and has been used in clinical and anthropological studies.[20,21]

The relevant criterion for the colour impression of PWS is the differential between the lightness of normal skin and of lesional skin. From this point of view the impression of the colour of a PWS is not only dependent on the absolute lesion colour but also on the skin type of the surrounding normal tissue.

One aim of the present study was to establish a simple and effective method for objective and quantitative evaluation of argon laser treatment results. With the digit numbers obtained by PC it is relatively simple to calculate per cent changes with treatment. With the CMR the large and the subtle lightness differences between PWS and normal skin were recognizable and even barely visible residual lesional skin after treatment could be evaluated by this method. We also tried to compare objective and subjective methods in the assessment of laser treatment of PWS. While median values of PC showed significant differences in the subjective categories, there was overlap among the different subjective categories as can be seen from the minimum and maximum values (Table 3). There was no significant difference regarding the relative median per cent blanching between the two groups that were divided into large and small mean lightness differentials. Objectively this would imply that clinical improvement with this treatment form is not necessarily dependent on colour intensity as has been previously suggested.[5]

Local application of heat to PWS showed neither a visual nor a measurable darkening of their colour. In contrast normal contralateral skin reacted with strong visible redness and a measurable decrease in lightness due to vasodilation. Our results suggest that under the given conditions ectatic PWS vessels are not capable of further vasodilation after external application of heat. Since an increase in the red colour tone in PWS is hardly recognizable by visual means, the advantage of PC is its ability to measure these subtle changes in skin lightness.

The disadvantage of PC is its unsuitability for differentiation between melanin and haemoglobin since an increase of either pigment leads to a decrease in lightness. Therefore it is difficult to document differentials in lightness of skin between different patients with varying skin types. This problem was avoided by measuring the normal contralateral skin area in each individual twice: at the first and at the final evaluation. Adjustments were made if necessary.

Most previous studies of objective assessment of PWS were done by reflectance spectrophotometry (RS), indicating a significant measurable difference between PWS and contralateral normal skin[12] and also between PWS colour before and after treatment.[10,11,13] Although more data can be obtained at many wavelengths by RS, the clinical value is limited because the different spectral reflection curves obtained by RS are hard to translate into human visual experience.[11] Because in PWS a basic colour and its various tones are always dealt with, determination of lightness by PC seems to be sufficient for objective evaluation of treatment.

Photoelectric colorimetry shows a good correlation with the visual evaluation of argon-laser-treated PWS but with greater reliability and reproducibility than would be possible using human observers. With this approach, it should be possible for different centres to assess and compare their treatment results. Similarly, different treatment approaches can be compared objectively. Extension of this method for other dermatological conditions remains to be evaluated.

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Wells' syndrome in children: varicella infection as a precipitating event

M.REICHEL, R.R.ISSEROFF, P.J.VOGT AND R.GANDOUR-EDWARDS
Departments of Dermatology and Pathology, University of California, Davis, School of Medicine, Davis, CA 95616, U.S.A

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Summary A case of Wells' syndrome is reported that developed in a 6-year-old boy 1 week after having chicken-pox.

Eosinophilic cellulitis or Wells' syndrome is a rare dermatosis with erythematous, urticarial plaques that become more indurated and eventually have a grey–blue discoloration. The histopathology is distinctive, with a diffuse infiltrate composed predominantly of eosinophils but admixed with lymphocytes, histiocytes, and occasional multinucleated histiocytes. There is oedema of the of the dermis with the so-called 'flame figures,' that consist of degenerated collagen focally enveloped with aggregates of eosinophil granules. These collagen fibres may be surrounded by palisading histiocytes and giant cells. Eosinophilia has been seen in the peripheral blood of half the cases. The course is variable, with waxing and waning, and eventual spontaneous resolution.

We report a case of a 6-year-old boy who developed Wells' syndrome 1 week after chicken-pox. Multiple factors, including infections, neoplasms, drug reactions, and insect bites have been invoked in the pathogenesis of Wells' syndrome. To our knowledge, varicella has not previously been implicated as a precipitating factor.

Case report

A 6-year-old Romanian boy presented to the University of California, Davis, Pediatric Dermatology Clinic complaining of a pruritic eruption of 6–7 months' duration. He was one of four children in his family and was otherwise healthy. The mother reported that 7 months previously all the children had chicken-pox and that in this child a new rash erupted the week after the chicken-pox resolved. The mother also reported that the child had hepatitis at the age of 3. No other member of the family had the rash.

Physical examination revealed an active, healthy boy with annular urticarial plaques with indurated borders and flat, central steel grey–blue discoloration (Fig. 1). The lesions were localized to the trunk, extremities, and face, sparing the knees, elbows, and scalp. There were no oral lesions. The lesions were in different stages of evolution, with the older resolving ones having a steel grey–brown hue. The clinical differential diagnosis at that time included erythema multiforme, urticarial vasculitis, and granuloma annulare.

Laboratory studies included a haematocrit of 36%, a WBC of 5700/mm^3 with 3% eosinophils, and an erythrocyte sedimentation rate of 5 mm/h. Blood chemistry and liver function tests were all within normal limits. Hepatitis B surface antigen, monospot, cold agglutinins, anti-hepatitis A IgM, and ANA were all negative. The herpes simplex virus titre was 1:128 and the varicella zoster virus titre was 1:32.

A skin biopsy revealed an intense superficial and deep dermal eosinophilic infiltrate which extended into the subcutaneous tissue (Fig. 2). Plasma cells were also present, most notably within the subcutaneous tissue. Within the dermis there were foci of eosinophilic degranulation and collagen degeneration consistent with flame figures. Macrophages and occasional multinucleated histiocytes were also present as well as scattered polymorphonuclear leucocytes. There was no significant alteration of the epidermis. A diagnosis of Wells' syndrome was made.

On follow-up 2 weeks later there was some resolution of the eruption and a significant improvement was noted after 1 month. A trial of triamcinolone acetonide 0·1% ointment was successful in clearing the remaining lesions. The child has been free of recurrent lesions for 4 months.

Correspondence: Dr Rivkah Isseroff, Department of Dermatology, TB 192 University of California, Davis, School of Medicine, Davis, CA 95616, U.S.A.

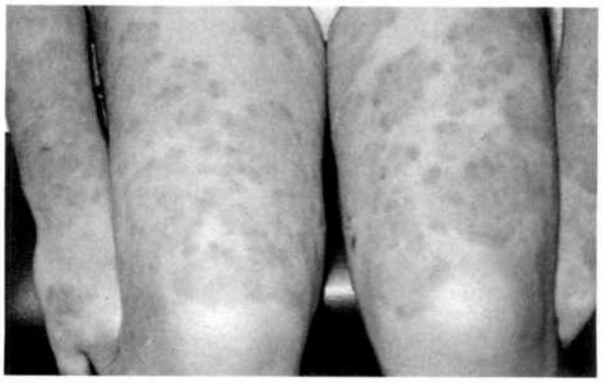

Figure 1. Annular urticarial plaques of Wells' syndrome.

Discussion

This child presented with the characteristic clinical and histological features of eosinophilic cellulitis or Wells' syndrome. He had a history of a febrile, vesicular illness that was consistent with chicken-pox 1 week prior to the onset of the urticarial lesions. A serum varicella zoster virus titre 6 months after the acute episode indicated a convalescent immunity. It seems likely that Wells' syndrome developed in this patient as a reaction to varicella.

The major histopathological findings of Wells' syndrome are an eosinophilic infiltrate with flame figures. Major basic protein, which comprises 50% of the protein in eosinophil granules, has been demonstrated in the flame figures of Wells' syndrome, showing that flame figures contain granules that have been extruded from eosinophils. Within the matrix of the eosinophil granule is eosinophil peroxidase. This generates free radical reactive oxygen species that are capable of destroying tumour cells and infectious organisms including viruses. Perhaps, in this case the varicella triggered an excessive cutaneous infiltrate with eosinophils. Massive degranulation can lead to tissue damage, with subsequent infiltration of the skin with phagocytes, and the formation of multinucleated histiocytes.

This is the sixteenth reported case of Wells' syndrome in a child.[1–10] Of the first two reported paediatric cases of eosinophilic cellulitis, one had a preceding case of mumps and the other of possible erysipelas.[1] Dermatophyte infection has also been reported in association with Wells' syndrome.[1] Wells' syndrome, like leucocytoclastic vasculitis, erythema multiforme, and erythema nodosum, represents a reaction pattern to a number of stimuli, including infections.[1,11,12]

Two cases of Wells' syndrome were reported in which there was a four-fold rise in the childrens' varicella zoster titre.[10] These patients had chicken-pox 1 and 3 years earlier, respectively. These changes in titres were attributed to non-specific immune stimulation. Wells' syndrome in these children may have developed as an aberrant immune response to rechallenge with antigen.

In adults, Wells' syndrome does not appear to have a gender predilection. In children, however, boys are predominantly affected (Table 1). This correlates well with other diseases which have an associated eosinophilia, in which males predominate. Two-thirds of patients with eosinophilic fasciitis (Schulman's syndrome),[13] over 80% of Japanese patients with angiolymphoid hyperplasia with eosinophilia (Kimura's disease),[14] and 90% of patients with the hypereosinophilic syndrome are males.[15] Eosinophilic pustular folliculitis

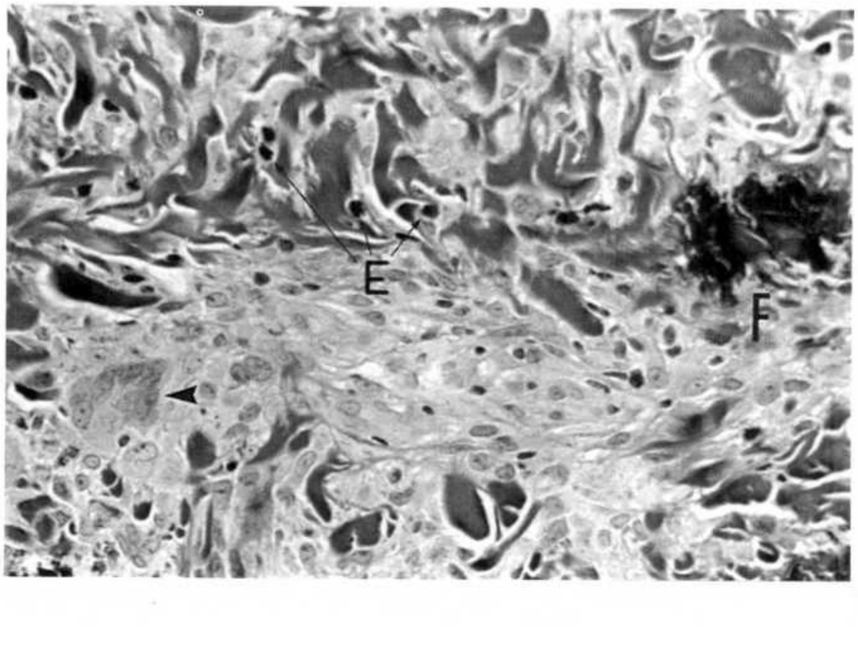

Figure 2. Dermal granuloma composed of histiocytes with a multinucleate giant cell (arrowhead), a flame figure [F], and eosinophils [E] (small arrows) (Haematoxylin and eosin, × 56).

Table 1. Wells' syndrome in children under 16 years

Author/year	Sex	Age (Years)	AEC*	Comments
Wells and Smith (1979)[1]	M	11	NR†	Mumps, penicillin
	M	12	11,440	Erysipelas, penicillin
Nielsen *et al.* (1981)[2]	M	11	5240	+ANA that reverted, atrophic alopecia
Bonvalet *et al.* (1983)[3]	M	2	800	
Saulsbury *et al.* (1983)[4]	M	7	1720	Oculomotor nerve palsy
Schorr *et al.* (1984)[5]	M	3·5	8500	Arthropod bite
Brehmer-Anderson *et al.* (1986)[6]	M	14	NR	
Wood *et al.* (1986)[7]	M	1·5	NR	
Kamani and Lipsitz (1987)[8]	M	7 weeks	2260	Familial. Pericarditis. Eosinophilic pulmonary consolidation.
	M	3 weeks	9280	Corticosteroid responsive
Correia and Garcia e Silva (1988)[9]	F	12	70	Diabetes mellitus
Lindskov *et al.* (1988)[10]	F	4	2225	Fourfold VZV titre increase
	F	5	26,000	Fourfold VZV titre increase
	M	1.5	2490	
	M	9	2405	Clinical impression: viral exanthem

* AEC, absolute eosinophil count, highest reported value.
† NR, not reported.

predominantly occurs in males.[16] Why diseases of eosinophils occur predominantly in males is unknown. Although oestrogen receptors have not been demonstrated on eosinophils, hormones may play a role. In one patient, exacerbations of Wells' syndrome occurred after parturition, premenstrually, and at ovulation; times during which there are high oestrogen levels or low progesterone levels.[17] In another case, oestrogens were felt to exacerbate Wells' syndrome.[18]

This case reports another male with Wells' syndrome, strengthening the male predominance of this disease in children. In addition, the association of antecedent chicken-pox suggests that varicella may be yet another precipitating factor for this syndrome. Several recent review articles report a total of 20–30 cases of Wells' syndrome.[6,10,12] We know of at least 71 cases.[1–12,17–30] Although this syndrome is more commonly reported in adults, it is much less so in children, this case representing the sixteenth report in a child younger than 16 years old.

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Autosomal dominant ichthyosis exfoliativa

F.VAKILZADEH AND G.KOLDE*

Department of Dermatology, General Hospital of Hildesheim, Hildesheim, Germany
* *Department of Dermatology, University of Munster, Munster, Germany*

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Summary An unusual ichthyosiform dermatosis with circumscribed areas of blistering hyperkeratoses as well as scaly areas was observed in five consecutive generations of a German family. Light and electron microscopical studies revealed oedema of the keratinocytes in the upper epidermis but no defects of tonofilaments and keratohyalin granules. We suggest that this type of ichthyosis exfoliativa inherited as an autosomal dominant trait represents a new and distinct entity.

Blistering is a rare event in the ichthyosiform dermatoses, but superficial blisters typically occur in cases of bullous ichthyosiform erythroderma that at birth have red, moist and tender areas of skin and later develop hyperkeratotic lesions and occasional blisters.[1] The other disorder of keratinization that is characterized with intraepidermal vesicles is ichthyosis bullosa of Siemens,[2] that was first described 50 years ago and was only recently observed in a second family.[3] We report what we consider to be a distinct entity of an ichthyosis with exfoliative blistering which showed clinical similarities to ichthyosis bullosa, but with distinct histological features.

Case reports

The ichthyosiform dermatosis was observed in four members of a German family. The pedigree of this family is shown in Figure 1 and the first case, her brother and her two children all had similar skin lesions. Her father, grandmother and great-grandfather as well as other relatives were known to have a similar ichthyosiform dermatosis.

Case 1

This 32-year-old woman was admitted with an ichthyosiform dermatosis that was present from birth. The patient had noticed an increased susceptibility of the skin to blister with trivial trauma. There were spontaneous blisters, especially during the summer and these blisters healed without scarring. On examination there were fine scales that covered the entire body apart from the face (Fig. 2). Dark grey hyperkeratotic areas were present on the waist, in the axillae and on the extensor and flexor surfaces of the elbows and knees, as well as the backs of the hands and the feet. At these sites there were occasional superficial blisters with denuded areas of skin. There were no abnormalities of the nails and hair. All the routine laboratory investigations were within the normal range.

Case 2

This 31-year-old brother of Case 1 had similar skin lesions since birth and had noted that the blisters became less frequent as he got older. The skin was dry and scaly all over (Fig. 3) apart from the face. Similar dark grey hyperkeratotic areas were present in this patient. The volar surfaces of the fingers had linear longitudinal hyperkeratoses. There was no other abnormality and all the laboratory investigations were normal.

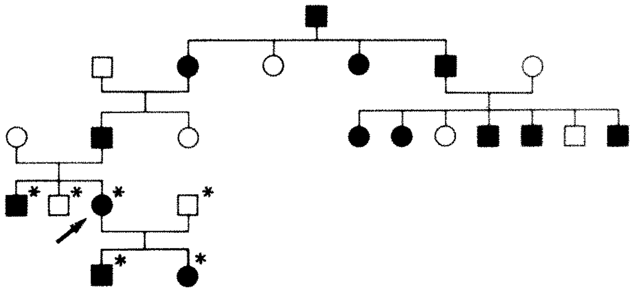

Figure 1. Pedigree showing transmission of ichthyosiform dermatosis in five generations. ■ affected male, ● affected female. → Case 1. * examined family members.

Correspondence: Dr Gerhard Kolde, Department of Dermatology, University of Munster, Von Esmarch Strasse 56, D-4400 Munster, Germany.

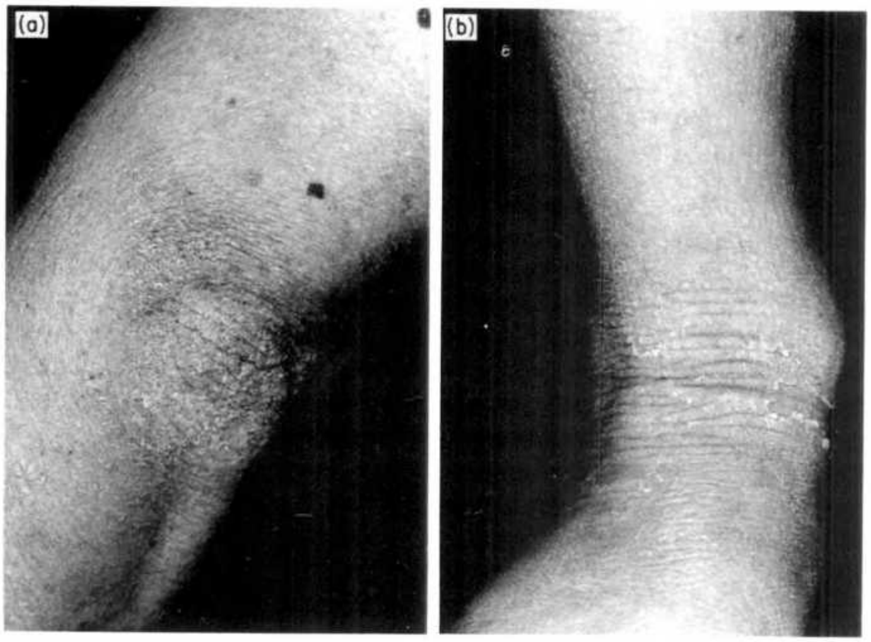

Figure 2. Case 1. Hyperkeratotic lesions with denuded areas on (a) the elbow and (b) the ankle.

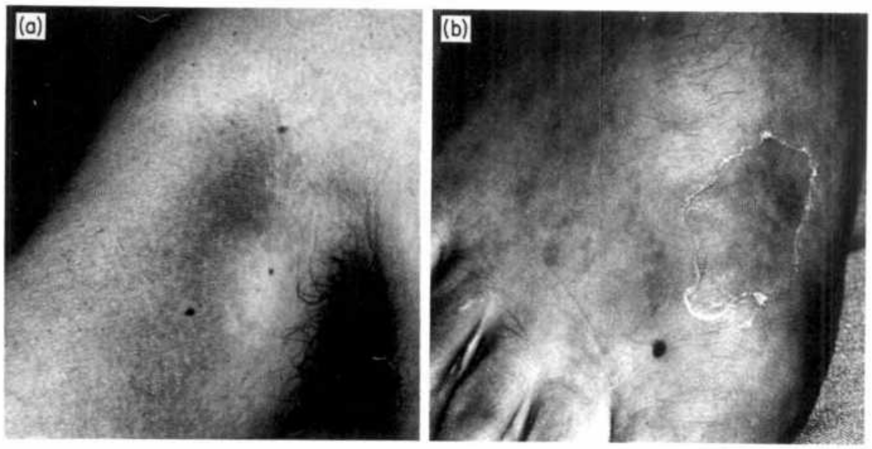

Figure 3. Case 2. (a) Circumscribed hyperkeratosis in the axilla. (b) Superficial blistering on dorsum of foot.

Case 3

The 13-year-old daughter of Case 1 had ichthyosiform dermatosis with increased fragility of the skin since birth. There was fine scaling of the skin with hyperkeratotic lesions in the axillae, on the elbows, knees and the backs of the hands and feet. There were numerous superficial blisters as well as denuded areas of the skin.

Case 4

The 6-year-old son of Case 1 had dry and scaly skin since birth. The hyperkeratotic lesions in this patients were less pronounced than any of the other cases, but had a similar distribution. Also multiple superficial blisters were seen in this case.

Histopathological studies

Multiple skin biopsies were obtained from Cases 1 and 2 and processed for light and electron microscopy. All the biopsies showed similar histopathological changes and there was no significant difference between those specimens taken from the hyperkeratotic areas and from the scaly skin.

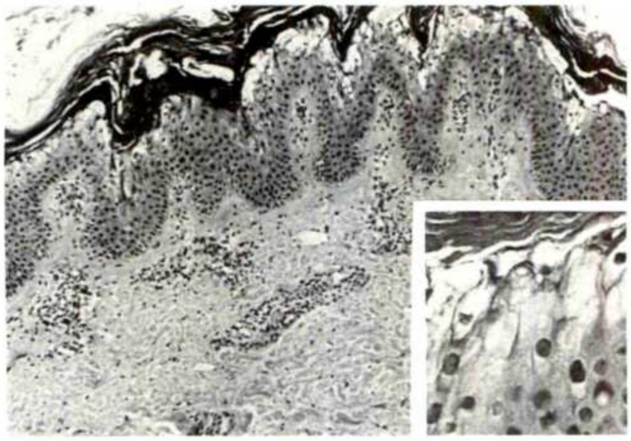

Figure 4. Skin biopsy of hyperkeratotic area showing markedly swollen keratinocytes in the upper spinous and granular cell layers (× 40). Inset: higher magnification showing oedematous keratinocytes (× 220).

There was marked acanthosis and hyperkeratosis of the epidermis (Fig. 4) and these were most pronounced in the hyperkeratotic areas. The keratinocytes in the granular and upper spinous layer showed marked oedema. The nuclei of the cells were small and hyperchromatic and the cells contained a few small keratohyalin granules. Due to the marked intracellular oedema, the cell boundaries often appeared to be indistinct. Small blisters were occasionally found directly beneath the horny layer. The basal and suprabasal keratinocytes appeared normal and there was no spongiosis. The mitotic activity of the epidermis was slightly increased. A few mononuclear inflammatory cells were seen in the upper dermis.

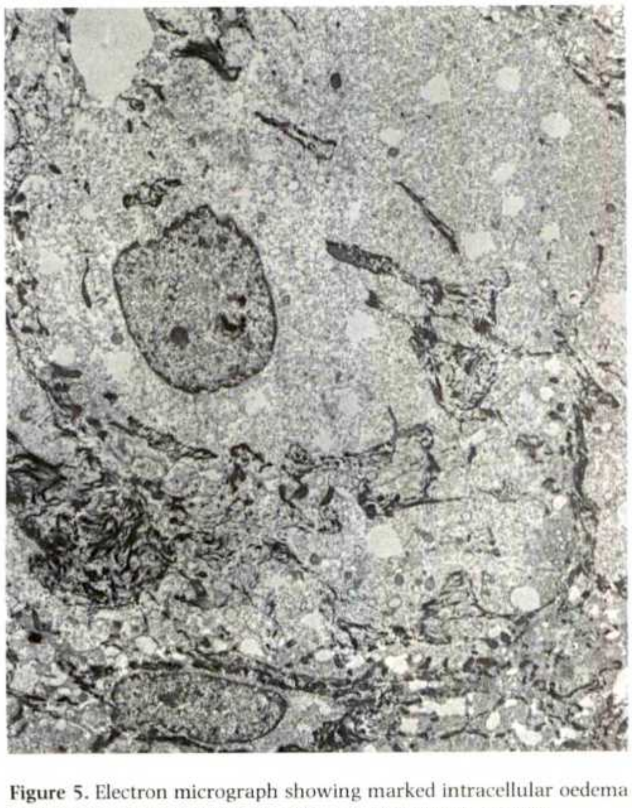

Figure 5. Electron micrograph showing marked intracellular oedema without any structural defect of the tonofilaments (× 6400).

On electron microscopy, the cells of the granular and spinous layer were characterized by marked oedema of the cytoplasm that contained few organelles and the small mitochondria were sometimes swollen (Fig. 5). The number of tonofilaments and keratohyalin granules were markedly reduced with no grouping of the tonofilaments. The few tonofilament bundles that were localized at the periphery of the cell showed a regular insertion into the desmosomal plates. Occasionally ruptured keratinocytes with fragmented plasma membranes were seen.

Discussion

The ichthyosiform dermatosis described in this report occurred in five consecutive generations of a family and this strongly suggests an autosomal dominant mode of inheritance. The skin lesions showed some resemblance to ichthyosis bullosa of Siemens,[2,3] and, as in this type of ichthyosis, there were dark grey hyperkeratotic areas in a generalized but circumscribed distribution with superficial blistering of the skin that occurred either spontaneously or after trivial trauma. However in our patient, in addition to these features there was a fine light scaling of the skin that resembled that seen in the autosomal dominant type of ichthyosis vulgaris.[1] In our patient the ichthyosiform dermatosis was present at birth and those cases as described by Siemens, developed the condition in the first year of life.[2]

The pathological features of ichthyosis bullosa of Siemens are unclear, but the blistering was reported to occur in the stratum corneum and the stratum granulosum of the ichthyotic skin.[2] In contrast the second report of this disorder[3] showed the typical histological and the ultrastructural features of epidermolytic hyperkeratosis with cytoplasmic oedema, thickened bundles of tonofilaments, clumps of irregular tonofilaments and coarse keratohyalin granules. These pathological changes are also found in bullous ichthyosiform erythroderma, which at birth has often generalized superficial blistering of the skin and later on the patient develops verrucous or hyperkeratotic skin lesions and occasional blisters.[1,4,5]

Because of the similarity of the pathological and clinical findings, we may speculate[6] that the dermatosis of Traupe *et al.*[3] represents a minor form of bullous ichthyosiform erythroderma.

The histopathological findings in our cases both from the hyperkeratotic and scaly areas showed the same features, and there was no clumping and 'shell' formation of the tonofilaments and no coarse keratohyalin as seen in the bullous type of ichthyosiform erythroderma[4,5] and the dermatosis of Traupe *et al.*[3]

In conclusion, our family with autosomal dominant ichthyosis exfoliativa show some clinical similarities to ichthyosis bullosa of Siemens, but the histopathological findings as seen on both light and electron microscopy suggest that it is different and a distinct entity.

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Chronic actinic dermatitis responding to danazol

P.HUMBERT, C.DROBACHEFF, M.VIGAN, E.QUENCEZ, R.LAURENT AND P.AGACHE

Department of Dermatology, Hôpital St Jacques, Besançon, France

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Summary We report an unusual case of chronic actinic dermatitis associated with α1-antitrypsin deficiency and who responded well to treatment with danazol.

Chronic actinic dermatitis (CAD) is a rare photosensitive disorder that usually affects elderly men.[1] The term describes a group of photosensitive dermatoses which include photosensitive eczema, persistent light reaction and actinic reticuloid.[1–4] The clinical, histopathological and photobiological features of these conditions that are characterized by marked light sensitivity continue to be a matter of debate because they are not well-defined disorders. The aetiology of CAD remains uncertain and the condition is usually refractory to treatment. Alpha-1-antitrypsin is an important regulatory protein involved in the suppression of a number of immunological and inflammatory processes. Its deficiency may be associated with marked immunological and inflammatory responses that result in the chronicity of various disorders.[5] We report a case of a patient with CAD associated with α1-antitrypsin deficiency who had a complete remission of the condition following treatment with danazol.

Case report

A 65-year-old man was seen in 1983 with a 10-month history of recurrent pruritus and redness of the face which then involved the forearms and the backs of his hands. The rash persisted throughout the year but was worse after sun exposure in the summer. The patient was not on any photosensitizing drugs. Over the previous 6 years he was admitted to hospital on several occasions because of exacerbations of the eruption (Fig. 1). The patient refused systemic corticosteroid therapy and immunosuppressive drugs but topical corticosteroids were helpful during exacerbations of the condition. There was no past history of dermatoses and no family history of any skin disease.

Light studies carried out using a Xenon arc lamp for UVB and Waldmann F 85 lamps for UVA showed a lowered minimal erythema dose for UVB (36 mJ/cm^2) and for UVA (7 J/cm^2). An exposure of more than 100 mJ/cm^2 of UVB produced papular and vesicular reactions. Photopatch tests with tetrachlorosalicylanilide, tribromosalicylanilide, bithionol, bronopol and fragrance mix all were negative. Routine patch tests were also negative except for thiuram mix and colophony.

The histopathological findings in multiple skin biopsies were those of an eczematous dermatitis. Direct

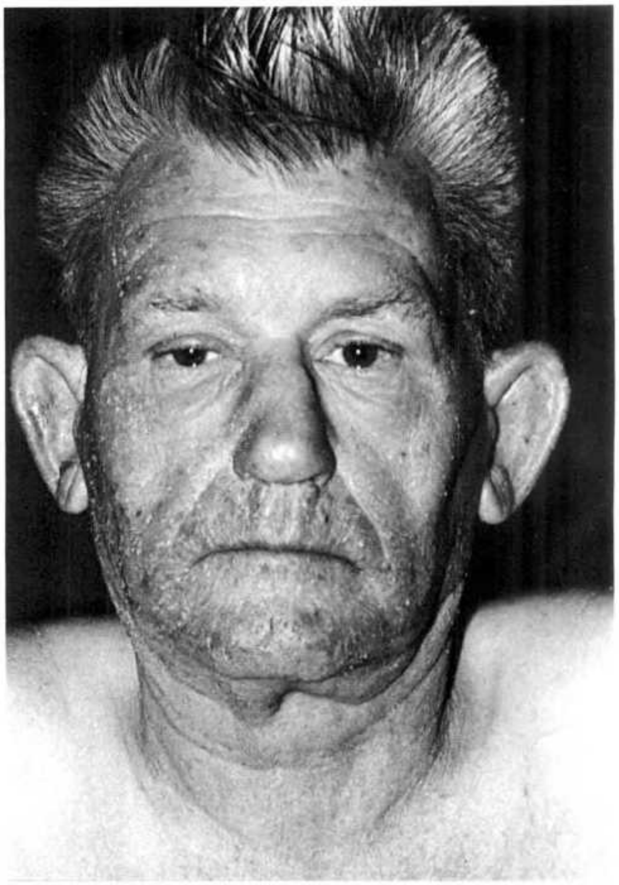

Figure 1. Chronic actinic dermatitis.

Correspondence: Dr P.Humbert, Department of Dermatology, Hôpital St Jacques, 25030 Besançon Cedex, France.

immunofluorescence showed deposits of IgG, C3 and fibrinogen in the walls of the blood vessels in the dermis.

In September 1988 he was readmitted to hospital and the α1-antitrypsin level was found to be 130 mg/dl (normal range 180–320 mg/dl). The phenotype using isoelectric focusing was PiSS. Other investigations were all normal.

Because of the failure of previous treatment and the severity of the condition, he was given danazol 600 mg daily. The condition rapidly improved and within 20 days the dermatosis had completely disappeared (Fig. 2).

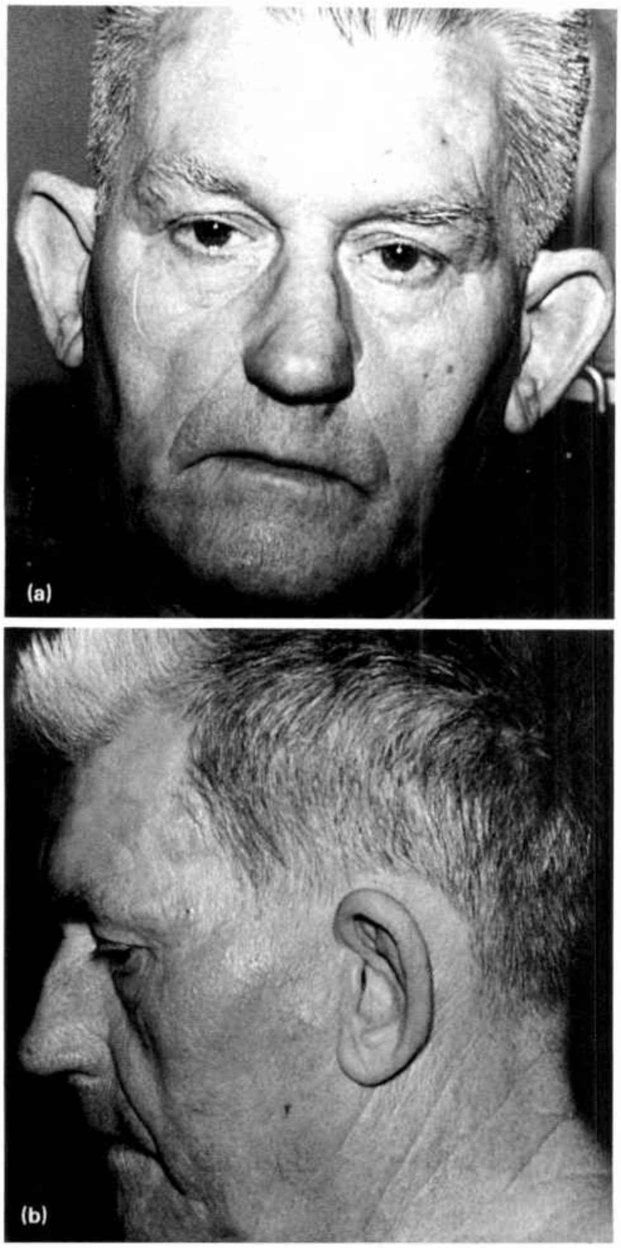

Figure 2. (a) and (b) Clearance after treatment with danazol.

Treatment was continued for 6 months and during this period he had no relapse. At that time the α1-antitrypsin level was 232 mg/dl. The patient then decided to stop taking danazol and within a few days there was a flare-up of the condition. On resuming treatment with danazol the condition cleared and he has continued to remain well after 18 months on this drug. So far no adverse effects of treatment have been observed.

Discussion

We report an unusual case of chronic actinic dermatitis in a patient with α1-antitrypsin deficiency. His symptoms and the quality of his life have been greatly improved since he was commenced on danazol.

Alpha-1-antitrypsin is a glycoprotein with a molecular weight of 52 kDa which is synthesized in the liver and consists of a folded polypeptide chain of 394 amino acids.[6] Over 30 different variants of α1-antitrypsin exist and are known collectively as the Pi system (protease inhibitor), the common variant being type M. The variant S is characterized by a single amino acid substitute in the primary chain.[7] In the homozygous form it is associated with α1-antitrypsin serum levels of 100–200 mg/dl[8] and experimental and clinical data suggest that deficiency of the antiprotease is associated with various abnormalities in the regulation of immune and inflammatory responses. Recent attention has been focused on the possible role of antiprotease deficiency in the pathogenesis of various cutaneous conditions and patients with chronic urticaria have been found to have significantly reduced levels.[9] In chronic urticaria/angio-oedema there is a reduction in inter-α trypsin inhibitor, and those with cold urticaria were found to have significant lowering of the level of α1-antichymotrypsin.[10] A number of dermatological diseases including persistent cutaneous vasculitis,[11] cold-contact urticaria and acquired angio-oedema,[12] psoriasis,[13] Ehlers–Danlos-like syndrome,[14] panniculitis[15] and pemphigus[16] may be associated with α1-antitrypsin deficiency. The chronic and marked inflammatory responses in patients with α1-antitrypsin deficiency may be due to the imbalance of protease–antiprotease. It could be argued that any drug that establishes homeostasis of the protease–antiprotease system would be beneficial to these patients. Danazol, an attenuated androgen, has been shown to produce a significant increase in the serum of patients with a deficiency of α1-antitrypsin.[17] Danazol has also been demonstrated to improve immunological and inflammatory conditions such as systemic lupus erythematosus, immune thrombocytopaenic purpura,[18] autoimmune haemolytic anaemia[19] and chronic urticaria,[20] cholinergic urticaria[21] and cholinergic pruritus.[22]

Recently immunosuppressive therapy has been reported to be of beneficial effect in CAD[23] and this suggests an immunological basis for this disorder. Alpha-1-antitrypsin deficiency could lead to increased T-helper cell activity and polyclonal B-cell activation.[5] These immunological abnormalities may be corrected by danazol therapy.

Alpha-1-antitrypsin deficiency is found in about 10% of the population in Europe[24] and these subjects are more predisposed to the development of inflammatory and immunologically mediated diseases. The search for α1-antitrypsin deficiency in patients with chronic inflammatory disorders of unknown aetiology is of importance and may lead to alternative treatment with such drugs as danazol.

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A case of classical mycosis fungoides associated with human T-cell lymphotropic virus type I

M.DETMAR, G.PAULI,* I.ANAGNOSTOPOULOS,† U.WUNDERLICH, H.HERBST,†
C.GARBE, H.STEIN† AND C.E.ORFANOS

*Department of Dermatology and † Institute of Pathology, University Medical Center Steglitz,
The Free University of Berlin, Germany
* Aids-Zentrum des Bundesgesundheitsamtes, Berlin, Germany*

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Summary

A 72-year-old male patient from north-eastern Iran developed the typical clinical and histopathological features of mycosis fungoides with lymphadenopathy, but without any other systemic involvement. Human T-cell lymphotropic virus (HTLV-I) antibodies were detected in the patient's serum by two different ELISAs and by Western blot using purified viral particles from MT-2 culture supernatants. Cultured peripheral blood lymphocytes were positive for labelling with anti-HTLV-I serum. Southern blot hybridization of DNA extracted from a skin tumour and from an involved lymph node revealed integrated proviral DNA with identical restriction patterns. This case supports a relationship between mycosis fungoides and HTLV-I and may indicate a new region of endemic HTLV-I infection.

The human T-cell lymphotropic virus type I (HTLV-I) is a geographically restricted human retrovirus associated with adult T-cell leukaemia/lymphoma,[1] spastic paraparesis[2,3] and chronic progressive myelopathy,[4] mainly confined to south-western Japan,[5] the Caribbean Islands,[6] south-eastern U.S.A.[7] and Africa.[8] We report a case of classical mycosis fungoides in an HTLV-I positive patient from north-eastern Iran, with detection of HTLV-I in cultured peripheral blood lymphocytes and detection of monoclonal proviral DNA in the tumour cells from a skin tumour and from an involved lymph node.

Case report

The 72-year-old male patient from Meschad, Iran who had never travelled abroad before, was admitted to the University Medical Center Steglitz, Berlin, with an 8-month history of disseminated, itching brownish-red plaques, papules and tumours of the skin (Fig. 1), accompanied by generalized lymphadenopathy. The patient had never received blood transfusions and denied other risk factors for HTLV-I infection. The full blood count, differential blood count, blood film, serum immunoglobulins and calcium levels were within the normal range. A chest X-ray, a bone scan, and a computerized tomographic (CT) scan of the abdomen showed no significant abnormality, nor did the histological examination of a bone marrow biopsy.

Several skin biopsies revealed the typical features of mycosis fungoides with epidermotropic infiltrates of small lymphocytes and the formation of Pautrier microabscesses (Fig. 2). Immunohistochemistry using the alkaline phosphatase–anti-alkaline phosphatase (APAAP) technique[9] revealed that the majority of infiltrating lymphocytes were stained by monoclonal antibodies against CD4, but not against CD8. In addition, most of the cells were positive for CD25, the interleukin 2 receptor. Electron microscopy showed typical mycosis fungoides cells with indented cerebriform nuclei within the epidermis. Investigation of several sections did not reveal any type C retrovirus particles. Infiltration of the paracortical zone by T-lymphoid blasts was found in a lymph node biopsy taken from the inguinal area. The infiltrating cells were positive for CD4, CD25 and Ki-1 (CD30) antigen, and negative for CD8.

HTLV-I antibodies were detected in the patient's serum by two different ELISA assays, one system established at our laboratory[10] and the other one kindly provided by Abbott (Chicago, IL, U.S.A.). The patient's serum (diluted 1:10) detected four viral proteins, p19, p24, p32, and p55, in Western blots of viral particles purified from MT-2 culture supernatants,[11] thus confirming the results of the ELISA (Fig. 3).

Correspondence: Dr M Detmar, Department of Dermatology, University Medical Center Steglitz, The Free University of Berlin, Hindenburgdamm 30, D-1000 Berlin 45, Germany

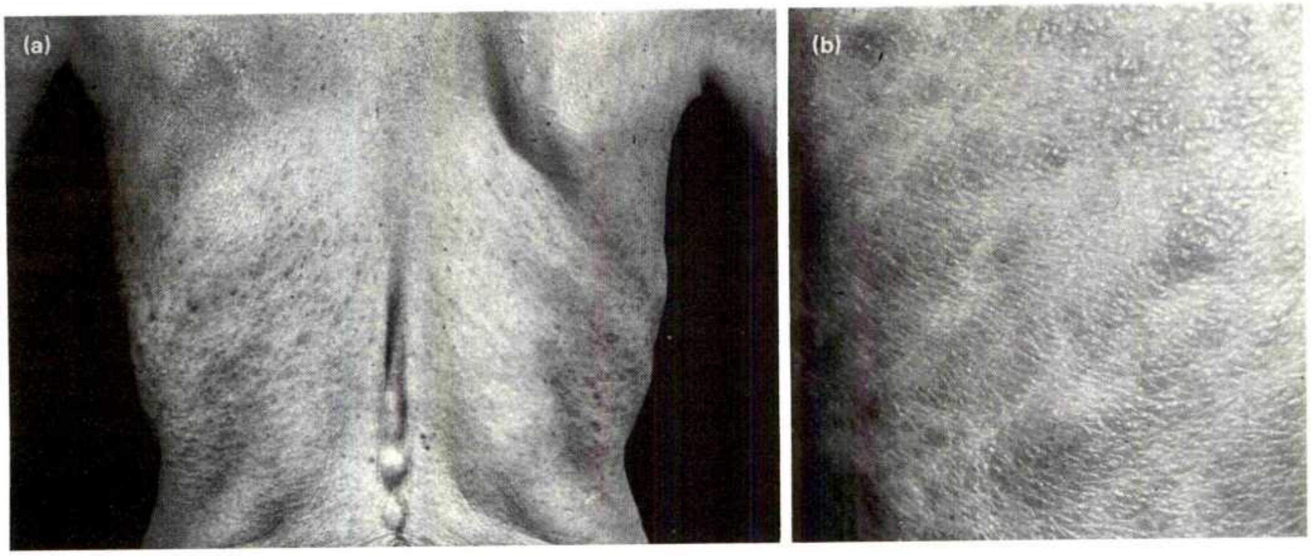

Figure 1. (a) and (b). A 72-year-old male patient with disseminated papules, plaques and tumours of the skin.

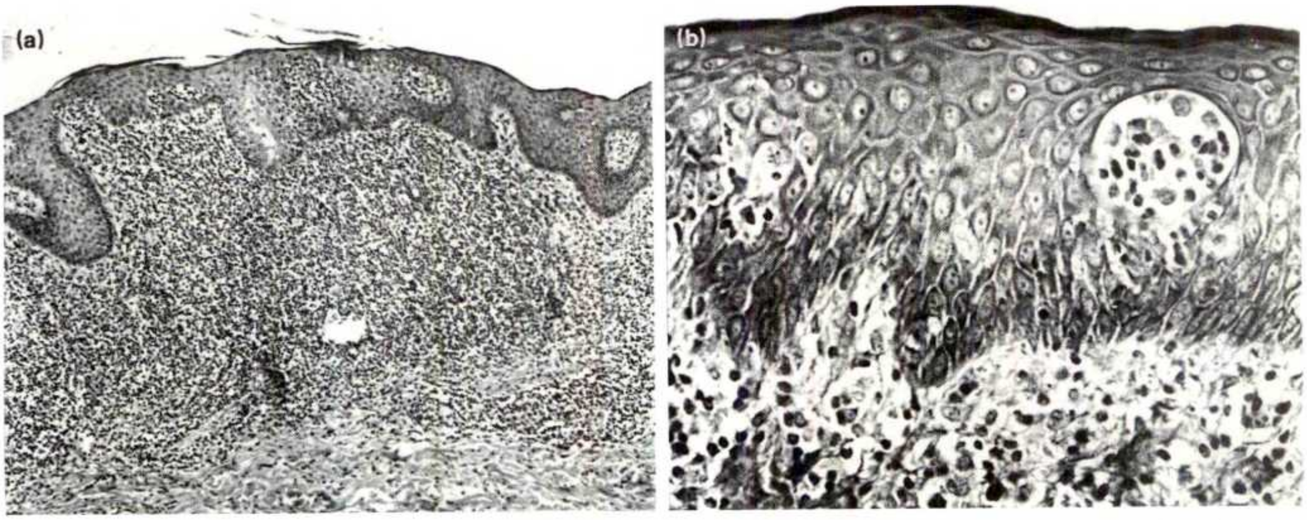

Figure 2. (a) Epidermotropic infiltrates of small lymphocytes. Haematoxylin and eosin. × 125. (b) Formation of intraepidermal Pautrier microabscesses. Giemsa staining. × 400.

Peripheral blood mononuclear cells were co-cultivated with cord blood cells[11] and a permanent cell line was established. Immunofluorescence assays using defined human sera with high titres of anti-HTLV-I, HIV-1, or HIV-2 revealed that co-cultured cells reacted with the HTLV-I serum only, showing a brilliant cytoplasmic fluorescence (Fig. 4). Non-infected cord blood cells were used as negative controls. Southern blot hybridization of DNA extracted from a skin tumour and from an involved lymph node using a ^{32}P-labelled HTLV-I probe (p23-3i,[12] kindly provided by Dr R.C. Gallo, Bethesda, MD, U.S.A.), revealed integrated proviral DNA with identical restriction patterns in EcoRI digests (Fig. 5), indicating a monoclonal origin of the infected cells at both locations.

The patient was treated with etretinate (50 mg daily) combined with photochemotherapy (PUVA) and with six cycles of polychemotherapy with monthly application of cyclophosphamide (300 mg p.o. daily for 5 days), vincristine (single dose of 2 mg i.v.), and prednisone (40 mg p.o. daily for 5 days), and achieved a complete remission of all skin lesions within 2 months of treatment. One year after initiation of the treatment, the patient still remains in complete remission.

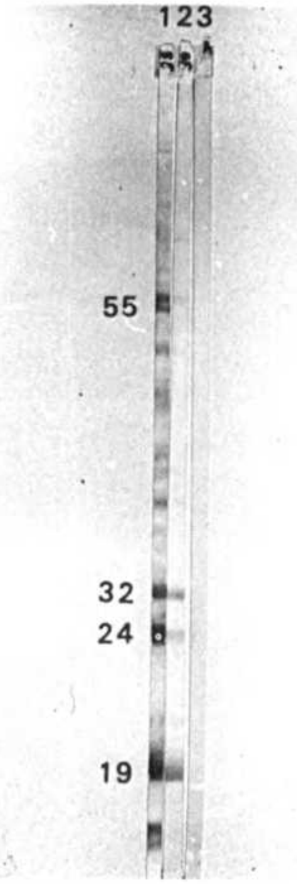

Figure 3. Western immunoblot of viral proteins reacting with patient's serum. 1, positive control serum; 2, patient's serum; 3, negative control serum. All tested at a dilution of 1:10. Detection of four viral proteins, p19, p24, p32, p55.

Discussion

This case showed the classical clinical and histopathological features of mycosis fungoides with lymph node involvement, representing cutaneous T-cell lymphoma stage IV, using the modified 'TNM' staging classification.[13] The vast majority of malignant lymphocytes in the skin lesions and in the lymph node were CD4$^+$. This is in accordance with previous reports on the immunophenotype of mycosis fungoides lesions.[14] In addition, the majority of the tumour cells expressed CD25, the interleukin 2 receptor. This has been rarely seen in classical mycosis fungoides, but has been consistently observed in HTLV-I-associated adult T-cell leukaemia/lymphoma (ATLL) caused by viral induction of protein synthesis.[15,16] The detection of HTLV-I antibodies in the serum, together with the positive virus culture confirms that the patient was infected with HTLV-I. Furthermore, the detection of monoclonal proviral nucleic acid sequences in cells from a skin tumour and from an involved lymph node provides evidence for monoclonal proliferation of the virus-infected lymphocytes in the characteristic mycosis fungoides lesions, and therefore suggests an aetiological role for HTLV-I in this disease.

It is of major interest that the clinical, haematological and histopathological features were not characteristic for either the acute or the chronic form of ATLL. The acute form of ATLL is characterized by disseminated disease, frequent bone marrow involvement, hypercalcaemia and a poor response to treatment,[17,18] while the chronic form and the 'smoldering' ATLL are characterized by circulating malignant cells with convoluted

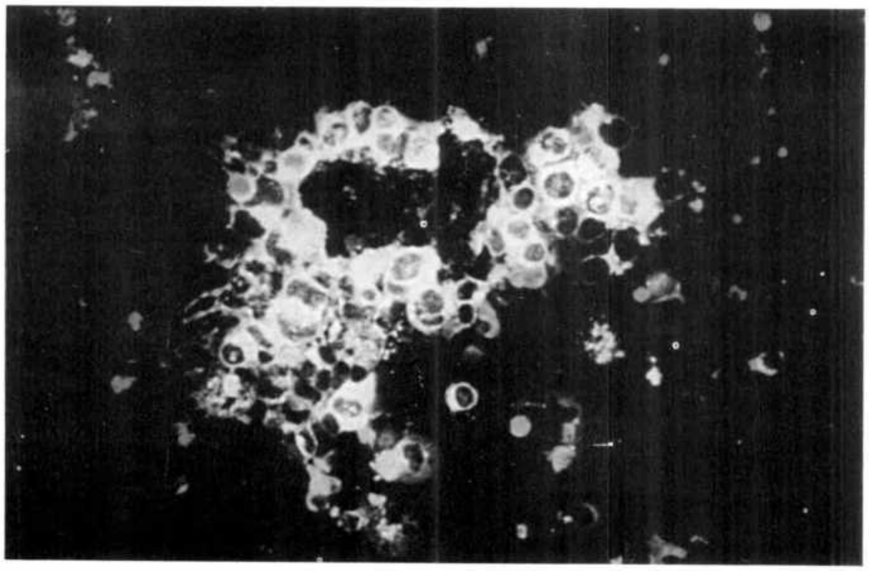

Figure 4. Intense cytoplasmic fluorescence after staining of patient's lymphocytes with human anti-HTLV-I serum. Lymphocytes were cultured in the presence of phytohaemagglutinin and interleukin 2 for 4 weeks.

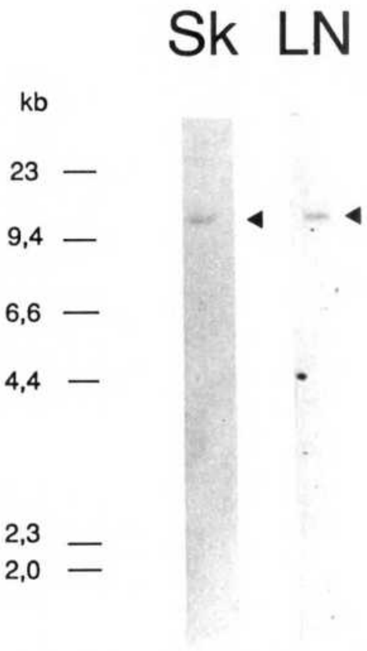

Figure 5. Southern blot hybridization of DNA extracted from a skin tumour (Sk) and a lymph node (LN), using HTLV-I probe p23-3i. Proviral DNA with identical restriction pattern in EcoRI digests.

nuclei (so-called 'flower cells'), and minimal skin involvement.[19,20] Lymphomas arising in ATLL are mainly of high grade malignancy, frequently containing lymphoid blast cells and only occasionally showing formation of Pautrier microabscesses.[21,22] These findings argue against the diagnosis of ATLL in our patient; however, we cannot completely rule out the possibility that this case may represent an atypical pre-leukaemic form of ATLL, as there are previous reports of patients with HTLV-I infection and longstanding skin lesions, who finally progress to ATLL.[23]

Two recent studies performed on patients with classical cutaneous T-cell lymphomas in Denmark and Germany[24,25] suggested that a minority of mycosis fungoides patients develop HTLV-I antibodies as evidenced by ELISA or by detection of p24 protein in Western immunoblots.[25] However, the rate of false positive results in the ELISA may be rather high,[10] and for the demonstration of an aetiological role of HTLV-I in cutaneous T-cell lymphoma it is essential to demonstrate clonal proliferation of the virus-infected tumour cells.[26]

The case presented strongly supports a viral aetiology in some cases of mycosis fungoides. The hypothesis of a viral aetiology gains further support by the reported detection of type C retroviral particles in skin and lymph nodes in patients with classical mycosis fungoides,[27] and by the recently observed good response of mycosis fungoides to intralesional and systemic treatment with recombinant interferon 2α.[28,29] It is conceivable that there are other viruses, possibly retroviruses, involved in the pathogenesis of cutaneous T-cell lymphoma that have not yet been detected by the presently used antibody assays. The case described here is, to our knowledge, the first report on HTLV-I positive cutaneous T-cell lymphoma in Iran and possibly indicates a new region with endemic HTLV-I.

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Acute febrile neutrophilic dermatosis in childhood (Sweet's syndrome)

P.COLLINS, SARAH ROGERS, P.KEENAN* AND MARY McCABE†

City of Dublin Skin and Cancer Hospital and St Vincent's Hospital, Dublin, Republic of Ireland
**St Joseph's Children's Hospital, Dublin, Republic of Ireland*
†Department of Pathology, St Vincent's Hospital, Dublin, Republic of Ireland

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Summary We report a case of Sweet's syndrome in childhood. Our patient suffered from repeated fevers, chest infections and had characteristic erythematous plaques. The severity of his illness increased with age and warranted oral steroid treatment. We review 16 cases reported to date in the literature and comment on the management and prognosis of the disease in childhood.

Case report

A 4-month-old male infant presented with a rash which his mother and family doctor said was present at birth. The skin lesions were distributed mainly on the cheeks and limbs, and consisted of well-defined, erythematous plaques and papules with occasional pustules (Fig. 1). It varied in severity over time and was unresponsive to dilute topical steroids. Histology showed regular epidermal hyperplasia with focal oedema of the papillary dermis. An inflammatory infiltrate consisting of large numbers of neutrophils, occasional lymphocytes and histiocytes was diffusely distributed in the mid-dermis (Fig. 2). There was marked leucocytoclasis but no vasculitis. At this time his haemoglobin was 9·5 g/dl and the sedimentation rate was 30 mm/h. Clinical and histological findings together suggested acute febrile neutrophilic dermatosis (AFND) or Sweet's syndrome.

At the age of 5 months the child was admitted to hospital with a 48-h history of irritability, cough, breathlessness, poor feeding and a temperature of 38°C. There were left basal crepitations and a chest radiograph showed a left basal pneumonia. His haemoglobin was 9·7 g/dl with microcytosis. The total white cell count was $15 \cdot 0 \times 10^9$/dl with a differential of neutrophils 59%, lymphocytes 33%, monocytes 5%, eosinophils 2% and basophils 1%. A sweat test to exclude cystic fibrosis was normal. He was treated with intravenous ampicillin and was discharged after 1 week. Three weeks later the child was readmitted with a right middle-lobe pneumonia. He had a leucocytosis but no neutrophilia. The serum IgG was reduced at 2·5 g/l (normal range 4·2–11·2), but IgM and IgA were normal at 0·71 g/l (0·32–1·30) and 0·42 g/l (0·21–1·26), respectively. On bronchoscopy there was pus in the trachea which yielded *Haemophilus influenzae* on culture. He responded to parenteral antibiotics. The rash flared during these two episodes but his systemic symptoms predominated.

Over the following year the patient had numerous chest infections which were treated with antibiotics. The rash became more extensive over this period. At 18 months he developed a plaque on his scrotum which rapidly extended to involve the napkin area. It was erythematous, indurated and vesicular, and extremely tender. Topical steroids and antibiotics were ineffective but oral prednisolone, at a dose of 2 mg/kg produced a dramatic improvement. The prednisolone was withdrawn gradually over a 2-week-period. The patient remained well for 4 months and his skin healed without scarring. He had two recurrences of his respiratory symptoms which were controlled with short courses of oral prednisolone.

At 2 years and 3 months his cough and breathlessness warranted readmission and the severity of his chest infection was mirrored by an exacerbation of his rash. Investigations at this time showed a right-upper and middle-lobe infiltrate on a chest radiograph. Bronchoscopy was unremarkable and aspirated mucus was sterile. The haemoglobin was 9·2 g/dl, white cell count $21 \cdot 7 \times 10^9$/dl, 71% neutrophils, 27% lymphocytes and 2% monocytes. Blood film showed a hypochromic, microcytic picture. Serum IgG was 4·6 g/l, IgM less than 0·32 g/l, IgA 0·77 g/l and IgE less than 2 IU/ml (0–100). There was an elevated C3 and C4, C3 1·48 g/l (0·55–1·2), C4 0·6 g/l (0·1–0·4) but a normal total haemolytic complement, CH50 46 (43–71, control 50/ml). There

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Correspondence: Dr P.Collins, City of Dublin Skin and Cancer Hospital, Hume Street, Dublin 2, Republic of Ireland.

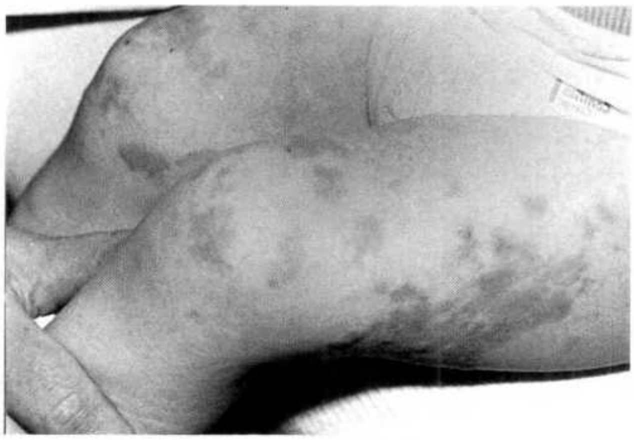

Figure 1. Erythematous plaques on thighs.

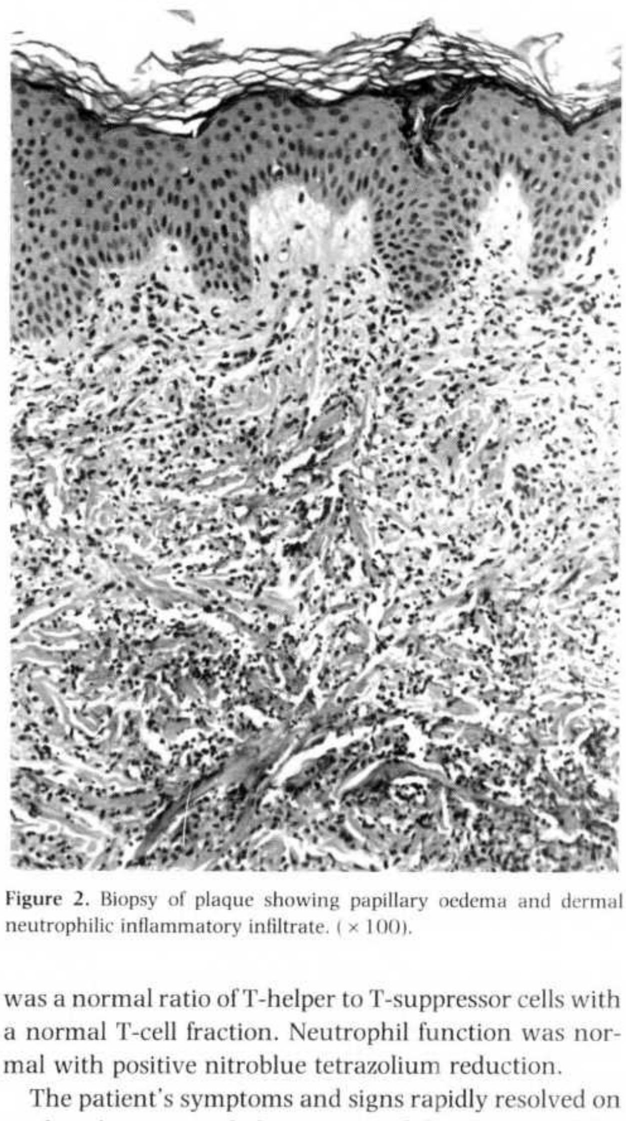

Figure 2. Biopsy of plaque showing papillary oedema and dermal neutrophilic inflammatory infiltrate. (× 100).

was a normal ratio of T-helper to T-suppressor cells with a normal T-cell fraction. Neutrophil function was normal with positive nitroblue tetrazolium reduction.

The patient's symptoms and signs rapidly resolved on prednisolone 5 mg daily. In view of the severity of the pulmonary infiltrate, the absence of pathogens and the frequency of the exacerbations it was decided to keep the patient on maintenance therapy of 5 mg prednisolone daily. At the time of writing, 4 months later, he remains well with significant clearing of his infiltrate. His haemoglobin is 12·5 g/dl, white cell count $8{\cdot}4 \times 10^9$ g/l with a normal differential and a normochromic normocytic picture on blood film.

Discussion

Acute febrile neutrophilic dermatosis (AFND) was described by Sweet in 1964.[1] The series included eight middle-aged females with fever, serum polymorphonuclear leucocytosis, painful plaques on the skin and a dermal infiltrate of polymorphonuclear leucocytes. The pathogenesis of this syndrome remains obscure. The blood vessels in the dermis show no evidence of damage in spite of the intense dermal neutrophilic infiltrate.[2] It may be a hypersensitivity reaction to bacterial, viral or tumour antigens. Studies of neutrophil chemotaxis, phagocytosis, and killing and metabolic activation have in general showed increased or normal levels of neutrophilic activity.[3]

Atypical features have been subsequently described in Sweet's syndrome including conjunctivitis, episcleritis, arthritis and arthralgia, myalgia, headache, gastrointestinal upset, anaemia and absence of neutrophilia.[3] Sweet's syndrome in adults has been associated with malignancy, predominantly haemopoietic, plasma cell and lymphoid[4] and two of the childhood cases had associated leukaemias (Table 1).

Eighty per cent of adult cases in the literature occur in females.[3] Unlike in adults, the paediatric cases show an equal sex distribution.[5-17] The age of onset in children ranges between 3 months and 12 years (Table 1). Whilst the clinical presentation of our case was consistent with Sweet's syndrome, he had a number of atypical features including the absence of neutrophilia and a persistent hypochromic, microcytic anaemia, which has resolved on steroid treatment.

Our patient's main problem has been respiratory. The failure to isolate significant pathogens from his chest and the absence of progressive lung damage on repeat bronchoscopy suggest clinically that he is immunocompetent. Immune function studies were normal apart from the levels of IgG and IgM. We suggest that the migratory radiographic changes represent neutrophilic infiltrates rather than consolidation. There has been significant improvement of his most recent chest radiograph while on maintenance prednisolone therapy.

Sweet treated his patients successfully with oral

Table 1. Sweet's syndrome in childhood

Reference	Age	Sex	Predominant features	Treatment
Klock[5]	10 years	M	Rash; leukaemia	Prednisolone local irradiation
Saxe[6]	9 months	F	Rash; tonsillitis	Antibiotics blood transfusions
Itami[7]	3 months	F	Rash	Antibiotics prednisolone
Levin[8] Muster[9]	17 months	F	Rash; respiratory Death	Prednisolone
Levin[8]	32 months	F	Rash	Prednisolone
Hazen[10]	10 months	M	Rash; otitis media	Prednisolone
Hazen[10]	9 months	M	Rash, respiratory	Dexamethasone prednisolone
Kibbi[11]	8 months	M	Rash	Antibiotics prednisolone
Attili[12]	24 months	F	Rash	Prednisolone
Edwards[13]	23 months	M	Rash; osteomyelitis	Prednisolone
Litt[14]	21 months	M	Rash; otitis media	Hydrocortisone antibiotics
Krilov[15]	3·5 years	M	Rash; leukaemia	Antibiotics prednisolone
Baron[16]	7 years	F	Rash; osteomyelitis; Fanconi's anaemia	Prednisolone
Baron[16]	12 years	F	Rash; Fanconi's anaemia; death	Prednisolone
Majeed[17]	19 months	M	Rash; osteomyelitis; anaemia	Dexamethasone
Majeed[17]	3 years	M	Rash; osteomyelitis; anaemia	Spontaneous remission
This report	4 months	M	Rash; respiratory; anaemia	Antibiotics prednisolone at 18 months

steroids apart from three, who were asymptomatic 2 months after presentation.[1] He considered that a given bout of the disease should remit spontaneously within several weeks to months. On reviewing the literature there appears to be a spectrum of disease activity in the paediatric cases reported. Some patients were acutely ill and required oral steroid treatment from the outset.[8,10,13] Until the age of 18 months our patient's rash was asymptomatic and was treated with topical steroid therapy. His chest symptoms were treated with antibiotics. Initially, oral steroids were given for the tender perineal plaque but ultimately steroid therapy was given for his pulmonary symptoms.

The prognosis in Sweet's syndrome depends upon the severity of the systemic disease which accompanies the rash. Sixteen children have been reported with the condition and their main clinical features and treatment are shown in Table 1. All presented with a rash. In addition, four had osteomyelitis,[13,16,17] two had chest disease,[9,10] two had otitis media[10,14] and one had a pancytopenia.[16] Thus, there is a broad spectrum of disease in Sweet's syndrome. The decision, if and when to give oral steroids should, therefore, be made with each exacerbation. Some children require maintenance steroids[8,16] whilst others have relapses not always requiring treatment. Two of the cases died, one of apparently unrelated causes[16] and the other, whose initial presentation involved her chest and her skin, died suddenly 2 years later from cardiac involvement.[8,9] Only two children have had respiratory disease and both required oral steroids. One is the child alluded to above who died because of cardiac involvement. The second child had severe subglottic stenosis and required tracheostomy for a few weeks but recovered on oral steroids.[10] He had exacerbations of his chest disease during the following year accompanied by the rash. The AFND settled after this but he was left with scarring (P.G.Hazen, personal communication).

The reluctance of a physician to treat an acutely ill child with fever, polymorphonuclear leucocytosis and chest infections with oral steroids is understandable and confirms the importance of early recognition of the syndrome and dual management by the paediatrician and the dermatologist.

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British Journal of Dermatology (1991) **124**, 207.

ADONIS 0007096391000466

Obituary

Dr Harold Twiston Davies

Harold Twiston Davies was regarded as unconventional by his skin specialist contemporaries, and with awe and affection by his junior staff and students.

Dermatologists in provincial non-teaching hospitals were almost non-existent before the inception of the National Health Service in 1948. Twiston Davies became honorary consultant dermatologist at the Royal Sussex County Hospital, Brighton, in 1926. This was 2 years after qualification following junior hospital appointments at Addenbrooke's, Cambridge, and the Hôpital St Louis in Paris, an international centre of dermatological excellence. He was fluent in French and German.

Twiston Davies was educated at Shrewsbury and served as a lieutenant in the Royal Flying Corps from 1916 to 1918. He survived a number of crashes and was invalided to the U.K. with gunshot wounds after a mission over enemy territory. After the First World War he went up to Jesus College, Cambridge, to study mechanical engineering and then changed to medicine. He completed his clinical course at St Bartholomew's Hospital in 1924.

His appointment as dermatologist to the Royal Sussex County Hospital, Brighton, gave him an abundance of untapped clinical material, as the only dermatologist in the south of England; an area now served by six consultants. Medical students and post-graduates with Brighton connections attended his out-patient sessions. His teaching was original and painstaking, and his clinical demonstrations memorable. He was oblivious to the fact that patients were waiting and that the nursing staff had a timetable to adhere to.

At the end of a busy clinic he might demonstrate the playing of a recorder to his students, or invite them to a round of golf which meant defeat unless they had a single figure handicap. In his private consulting room there was a notice stating his fee was three guineas which would be paid at the time of consultation. In 1940 Twiston Davies was commissioned into the RAMC, attaining the rank of lieutenant colonel, and was adviser in dermatology to the army in southern India. On demobilization in 1946 he resigned his Brighton appointment and became consultant to the Manchester Royal Infirmary, the Salford Skin Hospital, Chester Royal Infirmary and lecturer at Manchester University.

In the 1950s he was responsible for many of the abstracts of current literature which appeared in the *British Journal of Dermatology*. His succinct and deflationary comments gave a true perspective of papers which superficially appeared original and important. For example, a paper on Climatic Environmental Factors in the Aetiology of Skin Diseases received the abstract: 'It is axiomatic that the observer of natural phenomena should never take anything for granted; and this applies to the words that he uses. It is therefore not easy to credit a writer who spells hidradenitis with a 'y' with the habit of trying to find out about the things he does not understand or even the ability to recognize them as such'.

Dr Harold Twiston Davies

Before retiring in 1958 he was elected president of the British Association of Dermatologists and presided at the Cardiff Meeting. He was also a member of the American Academy of Sciences.

Fellows of the association complete a biographical record. In the section on publications, Twiston Davies records 'nothing of importance'. He was however, the author of over 70 publications, including original work on penicillin in infective conditions of the skin, industrial dermatoses, and cot deaths; this last in the era before the 1939 war.

Following his retirement from medicine in 1958 he bought and managed a 400-acre farm near Amersham, Bucks. Ten years later he moved to Carmarthen. He is survived by his wife, Carolina Marissa, and their two children, Caroline and Ceri, who is an orthopaedic surgeon; and by Elizabeth, John and Julian, the children of his previous marriage.

PATRICK HALL-SMITH

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Correspondence

Cross-over designs for clinical trials

SIR, Berth-Jones and Graham-Brown[1] compared terfenadine with placebo for pruritus in atopic eczema using a double-blind controlled cross-over design. Although it is easy to agree with their conclusion, we cannot place much reliance on the evidence that they presented, even though 'there was no evidence of a carry-over or period effect'. Although they quoted Hills and Armitage,[2] a masterpiece of clear exposition on the limitations of the cross-over designs and what to do about them, they went on to recommend strongly the use of the cross-over design.

The crux of the problem with a cross-over design is the emphasis on economy in the number of patients. The study is planned to use all the data collected from both periods of treatment in the analysis, with each patient contributing two sets of responses. It is hoped that the estimates of response to treatment will be more precise as 'each patient is his own control', and that therefore fewer patients (less than half the number required for a parallel group design) will be needed in order to come to a clear conclusion. Unfortunately the analysis of all the data so collected is only valid when there is no interaction of response to treatment with time, i.e. when those who start on Drug A show a response of size R_A to Drug A and a response of size R_B to Drug B, while those who start on Drug B show a response of size R_B followed by a response of size R_A on Drug A (on average), hence making sense of the consolidation of the two sets of results. Even when there should be no pharmacological reason for such an interaction (as in Berth-Jones and Graham-Brown's study, where it was expected that the pharmacological effect of the first drug taken would be long gone before the second drug was administered) a common finding is that the responses to a treatment differ in amount with the period of treatment, i.e. that there has been an interaction.

It is possible to test statistically for the presence of an interaction. Assuming that there has been no interaction, tests for evidence that an interaction exists are performed. The power of this test to show the existence of an interaction is based only on the number of patients (one response per patient) which is already low; thus it is not very sensitive, and may very easily return the result that insufficient evidence of an interaction exists. If the unwary investigator accepts that no evidence of an interaction means that there is no interaction, or, worse, does not test for an interaction at all, there must be occasions when an interaction not shown by testing is actually present to a degree sufficient to render the analysis of the full set of data invalid and hence misleading.

Hills and Armitage conclude: 'If an unequivocal result is required . . . a parallel group study should be carried out. If the number of patients is limited and a cross-over design is chosen, then the internal evidence that the basic assumptions of the cross-over are fulfilled must be presented and if necessary the conclusions should be based on the first period only'.

I feel that this seemingly attractive study design should only be used when no other design will serve.

Department of Dermatology M.F.CORBETT
Addenbrooke's Hospital
Hills Road
Cambridge CB2 2QQ, U.K.

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Reply

SIR, We thank Dr Corbett for her observations regarding the cross-over design used in our investigation into the value of terfenadine for relieving the pruritus of atopic dermatitis.[1] These observations could equally apply to many hundreds of other studies using this design. Although it has well recognized limitations which are described in the reference quoted in our report, the cross-over format remains popular and there are good reasons for this.

It is impossible to match patients with atopic dermatitis so that one is controlled by another. Not only is the disease very heterogeneous but so also is the way in which subjects use visual analogue scales, and the degree to which they demonstrate placebo response. The advantages of using each patient as his own control as in the cross-over design are therefore considerable.

We agree with Dr Corbett that the power of tests for carry-over or period effect is low, but the important thing is that there was absolutely no reason to anticipate that any such effect would occur in this trial. These are precisely the circumstances in which the crossover format is ideal.

Whilst accepting that no design is perfect, we therefore maintain that the cross-over format most is appropriate for studies of this kind.

Department of Dermatology J.BERTH-JONES
Leicester Royal Infirmary R.A.C.GRAHAM-BROWN
Leicester LE1 5WW, U.K. J.THOMPSON

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ADONIS 000709639100047J

Systemic TNF administration in psoriatic patients: a promising therapeutic modality for severe psoriasis

SIR, Recent evidence indicates that immunological mechanisms are involved in psoriasis and a variety of cytokines have been suggested in the pathogenesis of this disorder.[1] Tumour necrosis factor (TNF), previously thought to have a narrow range of biological activity, producing a haemorrhagic necrosis of tumours,[2] has now been found to stimulate a variety of cells that are involved in immune responses.[3] We have shown an absence of TNF as well as a decrease in the level of interleukin 1 in lesions of psoriasis.[4,5] In autoimmune animal models, replacement therapy with TNF has been shown to induce a significant delay in the development of lupus nephritis,[6] and to prevent diabetes in non-obese diabetic mice.[7]

In the present study we have evaluated the effect of systemically administered TNF, which has been used for immunotherapy of various malignant tumours,[8] in the treatment of patients with extensive psoriasis. The first was a 57-year-old male with a PASI score[9] of 40·6. Patient 2 was a 40-year-old female with a PASI of 24·9 and Patient 3 was a 28-year-old male with a PASI of 11·8. Both patients 1 and 2 had profuse lesions of psoriasis for many years (Figs 1a and 2a) and had not responded to the very potent corticosteroids administered. Patient 3 had lesions of psoriasis on the legs. The patients gave informed consent for the study and the treatment protocol was approved by our Medical Ethics Committee.

A prick test with TNF was performed using 12·5 U/ml to

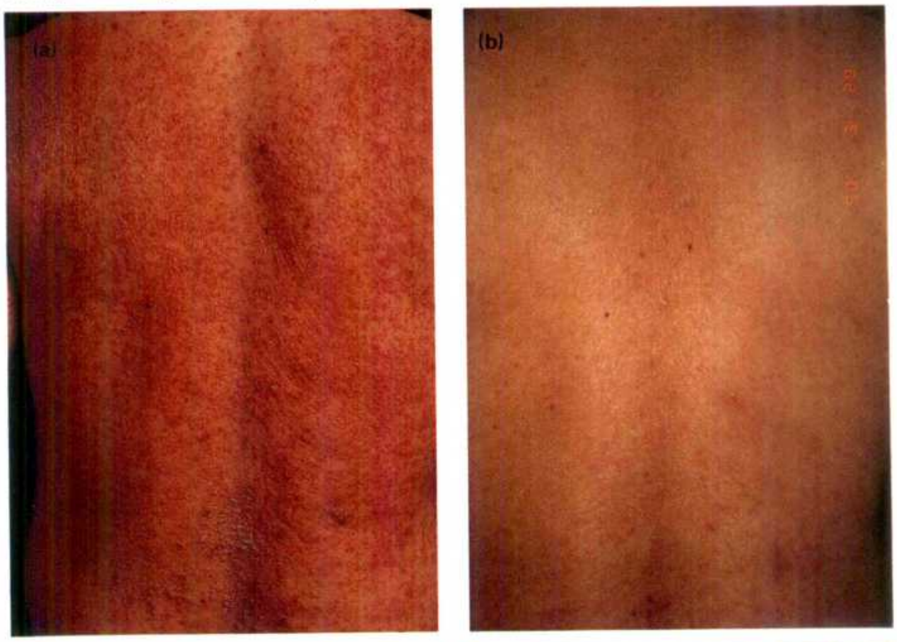

Figure 1. (a) Lesions of psoriasis on back of Patient 1 before treatment and (b) after third course of treatment with TNF.

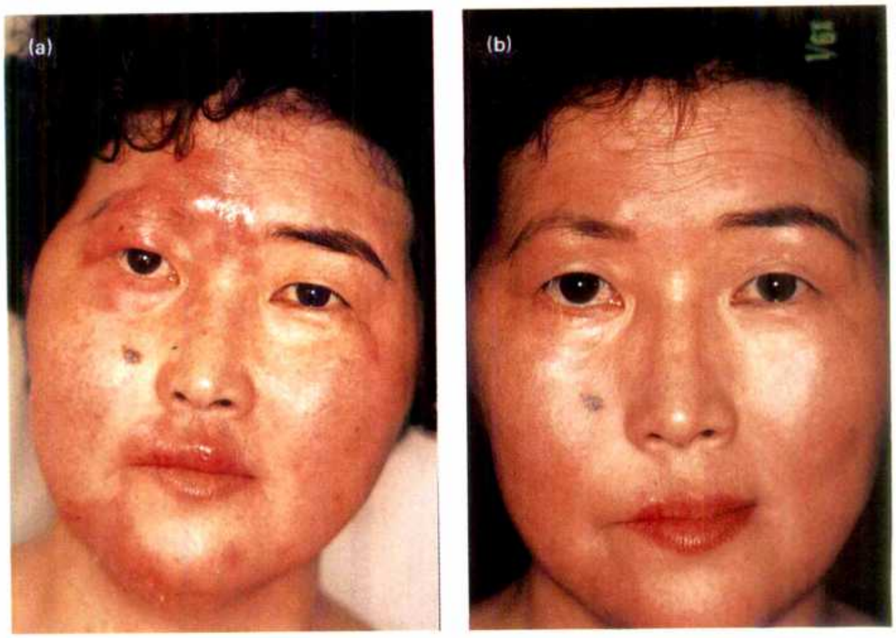

Figure 2. (a) Lesions of psoriasis on face of Patient 2 before treatment and (b) after third course of TNF therapy.

exclude a hypersensitivity reaction. Patient 1 received 2×10^5 U/m^2 body surface area of recombinant human (rH)-TNF (Asahi Chemical Industry, Tokyo, Japan) three times over 5 days. This course was repeated three times during a period of 2 months. The dosage of rH-TNF for Patients 2 and 3 was increased as follows: 1×10^5 U/m^2 for 5 days, 2×10^5 U/m^2 for 5 days, 5×10^5 U/m^2 for 5 days. There was a period of 2–3 weeks when there was no administration of the drug between each course.

A flattening of the lesions of psoriasis with decreased erythema and scaling were noted after the second course of rH-TNF administration in Patients 1 and 2. After the third course Patient 1 showed almost complete clearing of the lesions on the trunk and face as well as a significant improvement of those on the extremities, with a decrease of the PASI score from 40·6 to 8·3 (Fig. 1b). Patient 2 had a complete clearing of the psoriatic lesions even on the face (Fig. 2b). However in contrast, there was no significant change noted in the lesions of psoriasis in Patient 3.

Both patients with severe psoriasis responded well to treatment with rH-TNF. However, Patient 3 with more localized psoriasis did not improve with rH-TNF treatment. All the patients had a persistent psoriasis. The results suggest that TNF may be of benefit in the treatment of psoriasis recalcitrant to therapy, including pustular psoriasis. Our patients preferred treatment with TNF to other therapies and the only significant side-effect that we found was a transient and reversible elevation of the serum transaminases. A more detailed clinical study is in progress with a much larger number of patients to determine the efficacy of TNF in the treatment of psoriasis.

Department of Dermatology,
Tohoku University School of Medicine,
1-1 Seiryo-machi, Aoba-ku,
Sendai 980,
Japan

H.TAKEMATSU
H.OZAWA
T YOSHIMURA
M.HARA
A.SAKAKIBARA
J.OYAMA
H.TAGAMI

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ADONIS 000709639100048W

Palmar and plantar erythema due to infusion of sympathomimetics in pregnant women

SIR, We read with interest the letter of Morin *et al.*[1] who reported a case of a pregnant woman who developed patchy erythema of the palms following an infusion of albuterol.

We have previously reported[2] three cases of pregnant women with twins who in premature labour developed a lupus-like acral erythema after the infusion of salbutamol, another sympathomimetic drug. These patients had no other complaints and tests for antinuclear antibodies, including anti-RO-SSA antibodies, were negative. Direct immunofluorescence of the skin was normal, as were the findings on capillaroscopy. The erythema completely disappeared following delivery and after stopping the drug.

Peripheral vasodilatation due to sympathomimetic drugs is well known,[3] though many patients with asthma who have been treated with an infusion of β_2-sympathomimetic drugs have, as far as we know, not developed palmar erythema. We believe that these drugs may aggravate the vascular effects of oestrogens that are responsible for palmar erythemas in pregnant Caucasian women.[4] We agree with Morin *et al.* that β_2 adrenoreceptors may be present in glomus bodies. In our patients there was marked erythema on the palms and the soles as well as on the pads and nailbeds of the fingers and toes where glomus bodies are abundant.[5] This side effect of β_2-sympathomimetic drugs is we believe quite frequent, but rarely reported.

Dermatology Service,
Saint-Louis Hospital,
75010 Paris, France;
**Pasteur Hospital*
06000 Nice, France

P.REYGAGNE
J.P.LACOUR*
J-P.ORTONNE*

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ADONIS 000709639100049B

Homozygous variegate porphyria: revision of a diagnostic error

SIR, At the International Meeting on Porphyrins and Porphyrias in Rome in 1987, a case was presented under the title of 'Gunther-like syndrome in heterozygous harderoporphyria?'. This 36-year-old male had during the first few months of life a dermatosis that was characterized by vesicles and blisters in the sun-exposed areas of the skin. Between the ages of 1 and 4 years he had eight to 10 convulsive attacks and there was a delay in his speech and motor development. He had defects of the distal phalanges of the fingers of both hands (Fig. 1), a cicatricial alopecia and mental retardation. At the age of 25 there was a recurrence of the convulsive episodes and he was treated with chlorpromazine and benzodiazepine.[1] It was suggested by colleagues that further investigations could be carried out, and we report the most recent results on this case which are shown in Table 1. Protoporphyrin oxidase activity in the patient was 21% of the normal value, and was 52% of the normal value in his father and 60% in his mother. Both parents had reduced enzyme activity and it is likely that our patient was homozygous. We consider that in view of the findings our patient has homozygous variegate porphyria that is similar to other cases that have been reported.[2-5]

Table 1. Porphyrin concentrations in urine, faeces, erythrocytes, plasma and blister fluid

			Normal values
Urine	δ-ALA	2·48	<4·00 mg/24 h
	PBG	1·84	<2·00 mg/24 h
	Total porphyrins	0·55	<0·15 mg/24 h
	TLC: 8 COOH	16	10–20%
	7 COOH	14	1–2%
	6 COOH	8	1–2%
	5 COOH	11	2–3%
	4 COOH	51	60–80%
Faeces	Copro	58	<50 μg/g dry weight
	Proto	214	<110 μg/g dry weight
	Uro	10	<6 μg/g dry weight
	X porphyrin	69	<5 μg/g dry weight
Erythrocytes	Copro	30	<16 μg/100 ml eryth
	Proto	320	<60 μg/100 ml eryth
	Uro	ND	<7 μg/100 ml eryth
Plasma	Total porphyrins	0·080	<0·004 mg/l
Blister fluid	Total porphyrins	0·052	Not detectable

δ-ALA, δ-aminolaevulinic acid; PBG, porphobilinogen; Copro, coproporphyrin; Proto, protoporphyrin; Uro, uroporphyrin; 4 COOH–8 COOH, porphyrins with different carboxylic groups; TLC, thin layer chromatography; eryth, erythrocytes.

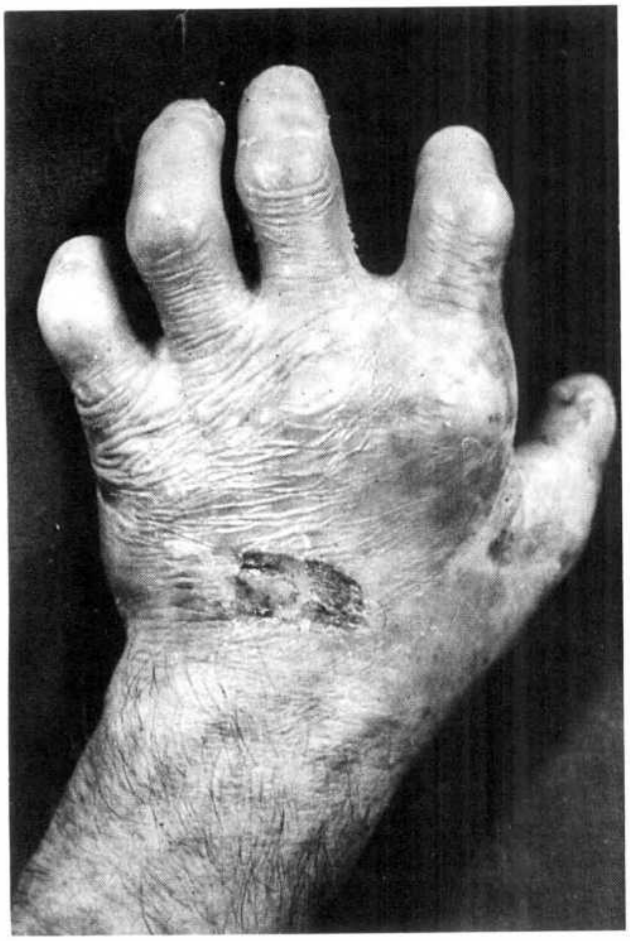

Figure 1. Claw-like appearance of hand of patient with hypoplasia of the distal phalanges and dystrophy of the nails.

Porphyria Center,
Istituto S.Gallicano,
Via S.Gallicano 25,
00153 Roma,
Italy
**Center Francais des Porphyries,*
Hopital Louis Mourier,
178 rue de Renouillers,
Colombes Cedex 927012,
France

L.D'ALESSANDRO GANDOLFO
A.MACRI
G.BIOLCATI
D.GRISO
L.N.PHUNG*
J.C.DEYBACH*
V.DA SILVA*
Y.NORDMANN*
G.C.TOPI

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ADONIS 000709639100505

Oral contraceptives and antibiotics in acne

SIR, The efficacy of the oral contraceptive pill in patients taking antibiotics[1] unfortunately remains unresolved. We have recently reported a possible case of contraceptive pill failure due to oral minocycline,[2] and have reviewed the literature on this controversial issue. We concluded that the risk of pregnancy attributable to the concomitant use of antibiotics appears to be very small, and suggested that these drugs can be safely prescribed. The patients should be made aware of the possibility, albeit remote, of a drug interaction. In another recent review the same conclusion was reached.[3]

The study of Drs Hughes and Cunliffe[1] certainly has obvious flaws. It is retrospective, involves only a small number of patients, and the results of questioning patients on past usage of antibiotics are not reliable unless they have a perfect memory. There were two pregnancies in an estimated 163 woman years of continued pill–antibiotic combination. This represented a sixfold risk of pregnancy when compared to a normal failure rate, which was said to be 2 per 1000 woman years (χ^2 test: $P<0\cdot05$). We doubt whether it is justified to draw firm conclusions from these data. Firstly, with patient-year experience it is advisable to estimate the relative risk with an incidence density ratio rather than using χ^2 statistics. With these data we came to a relative risk of 6·1 (95% CI: 1·1–34·0). Apparently, the effect is statistically only of borderline significance. Secondly, the authors did not interview a control group of women on contraceptive pills who had not used antibiotics concomitantly. The upper limit of the normal failure rate as quoted by the manufacturers of oral contraceptives is not necessarily the failure rate under everyday circumstances. The rate of unintended pregnancy has also been reported to be approximately 1%.[3] If this figure were to be used as normal failure rate, there would be no increased risk.

As they conclude that their results require further study, we feel that the advice against the use of oral antibiotics in acne subjects who are on oral contraceptives is premature. We fear that their advice may shift prescribing habits and result in the increased use of the teratogenic retinoids in women of childbearing age. Lawyers in the U.S.A., where 'suing your doctor' appears to have become a national sport,[4] may well be grateful for this letter, but dermatologists most likely will not.

Department of Dermatology,
Carolus Hospital,
P.O. Box 1101,
5200 BD 's-Hertogenbosch,
The Netherlands

A.C.DE GROOT
H.ESHUIS
B.H.C.STRICKER

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Successful treatment of alopecia areata using the contact allergen diphencyprone

SIR, Diphencyprone (diphenylcyclopropenone-1) has been used successfully in Leeds for the treatment of alopecia areata (AA) and alopecia totalis (AT) for 3 years.

Currently we have 78 adult patients (17 male, 61 female) who have been treated with diphencyprone (DPC) for 8 months or longer. All patients recruited have had alopecia for a minimum of 6 months; the average duration of alopecia was 7 years (range 0·5–35 years). Forty-five of the patients presented with AT and 33 with AA. After 8 months of weekly therapy, 29 patients (22 AT, seven AA) showed no growth, but 49 (23 AT, 26 AA) showed regrowth of hair. Twenty-five of these 49 achieved complete regrowth of scalp hair (18 AA, seven AT). Eighteen of the 49 have good regrowth of scalp hair which is continuing to progress, and six have some regrowth ranging from sparse terminal hairs to patchy regrowth. Therefore, after a minimum of 8 months treatment with DPC 62% of patients showed regrowth; 32% had complete regrowth, 23% had good progressing regrowth and 8% had some regrowth.

It would seem therefore, that in our hands DPC is a useful treatment for AA and AT. It continues to surprise us that our results are not mirrored in other centres.[1] A possible explanation for the differences in response to DPC between various centres is patient selection; however, there was no significant difference in the duration of alopecia for those that regrew (mean 8 years) and those that did not (mean 10 years), upholding the observations made on our original cohort.[2]

Diphencyprone is obtainable commercially as a pure substance. However, each batch of the compound is analysed for impurities, in particular for the presence of the mutagenic precursor $\alpha.\alpha_1$dibromodibenzylketone. Diphencyprone is stable and when in solution with acetone has a shelf-life of 3 months, provided that it is protected from UV-light by storage in amber glass bottles. All patients must be instructed to keep their heads covered for a minimum of 6 h post-therapy and advised not to wash their heads for 48 h after the application of DPC.

Sensitization is achieved by applying a 2% solution to a small area on the scalp (4-cm diameter), and if this does not result in adequate sensitization the process is repeated beneath plastic occlusion. Thereafter, weaker concentrations of DPC are applied (0·01, 0·025, 0·05–2%) and gradually increased in order to elicit erythema and pruritus in the area of treatment for 36 h post-therapy. Only one side of the scalp is treated until the optimum concentration is found, to avoid the possibility of a widespread adverse reaction. Treatment is weekly.

Signed informed consent is given by all patients. The need for adequate contraception is stressed and treatment does not start until a negative pregnancy test is received for patients of child-bearing age. Haematological, hepatic and renal function screening are performed at 6-monthly intervals. Each patient is given a topical steroid for use in the event of a florid reaction developing.

We have found that provided patients are fully informed of the weekly commitment required and all possible side-effects of treatment, they tolerate the treatment with ease.

Department of Dermatology,
General Infirmary at Leeds,
Leeds LS1 3EX, U.K.

S.MACDONALD HULL
W.J.CUNLIFFE

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British Journal of Dermatology (1991) **124**, 214.

ADONIS 000709639100053A

Book Review

Atlas of Cutaneous Facial Flaps and Grafts. A Differential Diagnosis of Wound Closures. RONALD L. MOY (1990) Pennsylvania: Lea & Febiger. Pp. 223. Number of illustrations 205. ISBN No. 0-8121-1313-6. Price $65.00.

This book, written by an American dermatological surgeon, is aimed at American dermatology residents. Given that not all U.K. dermatology registrars are as enthusiastic surgeons as their American counterparts the market may be smaller in this country. Apart from the first 18 pages the book consists entirely of series of colour photographs and black and white line drawings showing a range of flap and graft repairs after cancer surgery on principally the nose but also the cheek, lip, temple and ear.

The book is not well set out, sections run into each other with no clear divisions, in at least two cases figures (Figs 30 and 31 and Figs 180 and 181) have been transposed and there is no index. Illustrations appear on one page with a brief legend on the opposite; hence there is a lot of wasted white space with plenty of room for either more pictures or a better description of the technique being illustrated. Line drawings, done by the author, are used when a suitable photograph of that particular stage is not available. They are unfortunately not always very enlightening and sometimes fail to complement the figures. In some cases the illustrations do not show the procedure in sufficient detail for the reader to be confident about each stage. Most of all I missed a clear step by step accurate description of the technique giving details of undermining, important anatomical landmarks and hazards to be avoided. Given the mass of empty space this could easily be added to enormous advantage.

The good points are the relevant subject matter and the honest way in which results have been shown so that not just the author's best results but also his less than perfect ones have been illustrated with methods of dealing with the problem. The book should have been set out better and more detailed legends would have helped enormously. There are several books currently available for the description of flap repairs. Compared to the *Practical Manuals in Dermatologic Surgery* series edited by Dr Roy Grekin this is a definite second choice but if you already have the Salasche and Grabski book on flap repairs on the nose and lip and want clinical examples of the repairs Dr Moy's book would be a useful addition. The idea is sound, the content adequate but the design and description inadequate and I hope they will be improved in the next edition. At the U.S. price of $65 it looks like a bargain but in the U.K. it will surely, but inexplicably, be much more and may seem overpriced for the material covered.

C. LAWRENCE

British Journal of Dermatology (1991) 124, 215–216.

News and Notices

Institute of Dermatology and Section of Clinical Immunology and Allergy, Royal Society of Medicine London, 10 June 1991

A 1-day symposium entitled 'Immunology of Skin Disease: New Developments' will be held at the Royal Society of Medicine, 1 Wimpole Street, London W1 on 10 June 1991. The faculty is international and will cover new developments in the basic science, clinical and therapeutic fields. For further details, please contact: the Dean of the Institute, Professor Malcolm Greaves or the courses' administrator, Miss Tessa Brewer at the Institute of Dermatology, St Thomas's Hospital, Lambeth Palace Road, London SE1 7EH. Telephone 071-928-9292, extension 8075 or 3314.

British Association of Dermatologists Awards for 1991

B.A.D. Travelling Fellowships
The B.A.D. has once again made available a limited number of Travelling Fellowships for Senior Registrars or Consultant Dermatologists. It is anticipated that successful Fellows would visit various centres in a country overseas, and should attend that country's national dermatological meeting. Fellowships to a maximum value of £1000 will be awarded for a period of not less than 3 weeks. Further details and application forms are available from the B.A.D. Office and completed applications must be returned to the Honorary Secretary by 15 March 1991.

Dowling Travelling Fellowship
Applications are invited for this Fellowship, which is tenable for up to 2 years. Applicants should be registered medical practitioners who are British graduates and who hold a higher medical qualification. The aim of the Fellowship is to encourage evident merit in Senior Registrars and Consultant Dermatologists or individuals of equivalent academic status and is intended to assist them to travel abroad to undertake study and research. The maximum value of this Fellowship is £10,000 p.a. Further details and application forms are available from: the B.A.D. Office and completed applications must be returned to the Honorary Secretary by 15 March 1991.

Gist–Brocades Fellowship
Applicants for this Fellowship should be at a relatively late stage in their dermatology training (preferably after achieving accreditation) and the Fellowship should be considered as a secondment from an existing Senior Registrar or equivalently graded post in the U.K. The Fellowship can be held only in a University or teaching hospital in the U.K. and is for 1 year only; its maximum value is £28,000. Further details and application forms are available from: the B.A.D. Office and completed applications must be returned to the Honorary Secretary by 15 March 1991.

Stiefel Travelling Grants
Applicants are invited for these Travelling Grants, which are intended to help Registrars, Senior Registrars and Consultant Dermatologists, or those with similar academic status working in dermatology, to attend international and national meetings abroad. Applicants must provide evidence of active participation in the meeting. Further details and application forms are available from: the B.A.D. Office and completed applications must be returned to the Honorary Secretary by 18 October 1991.

Study Fellowships
Two Study Fellowships are available for Senior Registrars or Consultants who wish to study or participate in research activities *in this country* for a period of up to 3 months. The value of each Fellowship will be up to £3750 and one shall be known as the Neutrogena Study Fellowship. Further details and application forms are available from: the B.A.D. Office and completed applications must be returned to the Honorary Secretary by 15 March 1991 and 18 October 1991.

The Wycombe Prize
Applications are invited for this prize, which is offered for an outstanding contribution to dermatology published from a district general hospital or non-teaching centre in the U.K. Further details and application forms are available from: the B.A.D. Office and completed applications must be returned to the Honorary Secretary by 15 March 1991.

6th Course in Paediatric Dermatology, 9–10 May 1991, University of Liverpool

A 2-day course on Paediatric Dermatology will be held at the University of Liverpool on 9–10 May 1991. This is for recently appointed Consultants and Senior Registrars and is limited to 12 participants. Further details are available from: Dr J.L. Verbov, c/o Postgraduate Office, Faculty of Medicine, P.O. Box 147, Liverpool L69 3BX. Telephone 051-709-3114.

6th International Congress of Pediatric Dermatology, Toronto, Canada, 8–11 June 1992

The 6th International Congress of Pediatric Dermatology will be held in Toronto, Canada on 8–11 June 1992. For further information contact: James E.Rasmussen MD, Department of Dermatology, University of Michigan, 1910 Taubman Center 0314, Ann Arbor, MI 48109–0314, U.S.A. Telephone (313) 936–4086, FAX (313) 936–6395.

3rd International Course on Occupational Dermatoses, 3–7 June 1991, Mariehamn, Finland

The 3rd International Course on Occupational Dermatoses will take place on 3–7 June 1991 at Mariehamn, Aland, Finland. For further information, please contact: Dr Lasse Kanerva, Institute of Occupational Health, Section of Dermatology, Topeliuksenkatu 41 a A, SF-00250 Helsinki, Finland. Telephone 358-0-474-7288; Fax 358-0-413-691.

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The *British Journal of Dermatology* publishes original articles on all aspects of the normal biology, and of the pathology of the skin. Originally the Journal, founded in 1888, was devoted almost exclusively to the interests of the dermatologist in clinical practice. However, the rapid development, during the past twenty years, of research on the physiology and experimental pathology of the skin has been reflected in the contents of the Journal, which now provides a vehicle for the publication of both experimental and clinical research and serves equally the laboratory worker and the clinician. To bridge the gap between laboratory and clinic the Journal publishes signed editorials reviewing, primarily for the benefit of the clinician, advances in laboratory research, and also reviews of recent advances in such aspects of dermatology as contact dermatitis, therapeutics and drug reactions. Other regular features include book reviews, correspondence and society proceedings. The Journal is the official organ of the British Association of Dermatologists and also of the Netherlands Association for Dermatology and Venereology, but it attracts contributions from all countries in which sound research is carried out, and its circulation is equally international.

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gram(s)	g
kilogram(s)	kg
milligram(s) (10^{-3}g)	mg
microgram(s) (10^{-6}g)	μg
nanogram(s) (10^{-9}g)	ng
picogram(s) (10^{-12}g)	pg
hours(s)	h
minute(s)	min
centimetre(s)	cm
second(s)	s
cubic millimetre(s)	mm^3
millimetre(s)	mm
millicurie(s)	mCi
milliequivalent	mmol
molar	mol l
millilitre(s)	ml
gravitational acceleration	*g*
micrometre(s)	μm
per cent	%
isotopic mass number placed as	^{131}I

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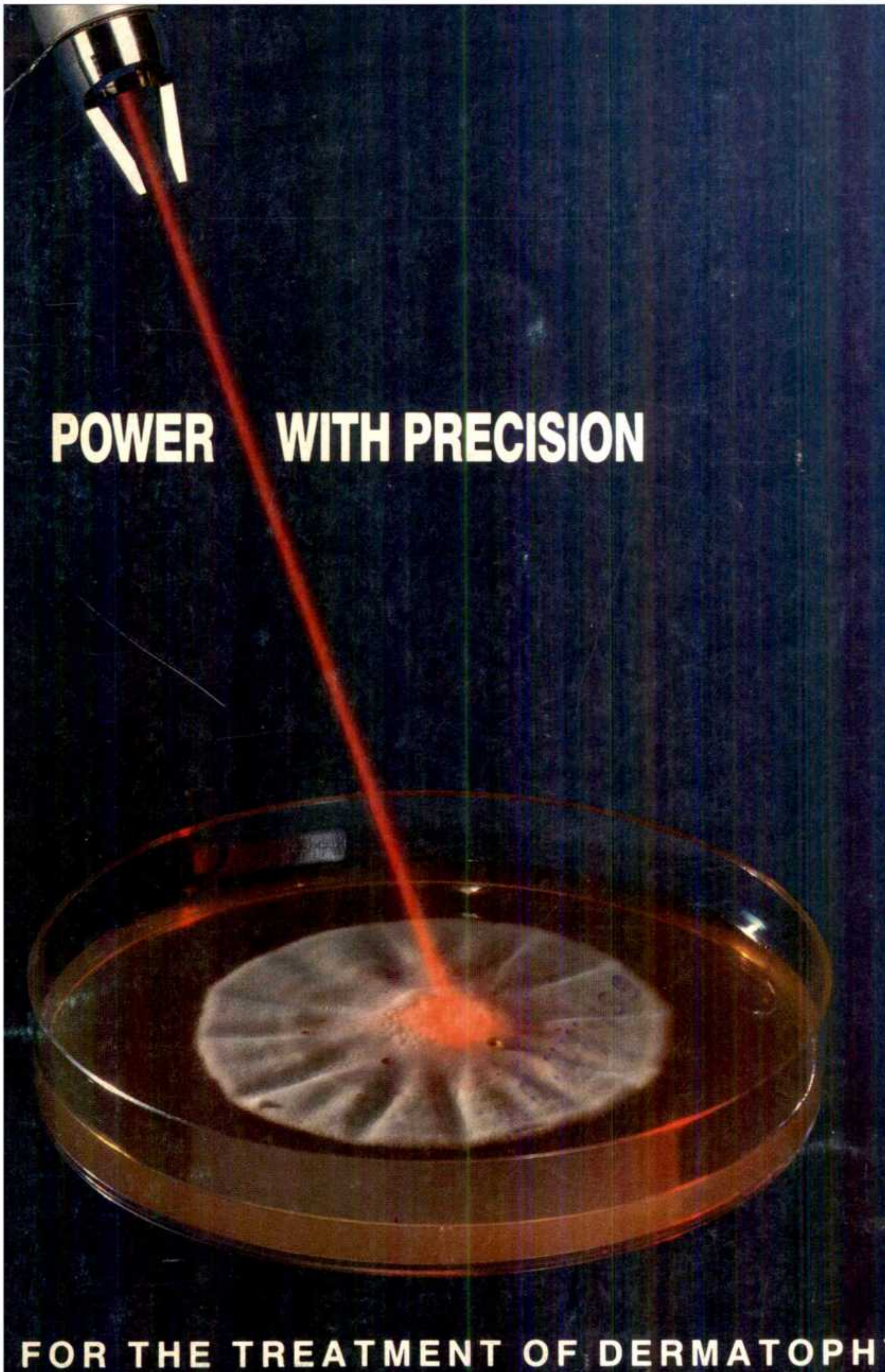

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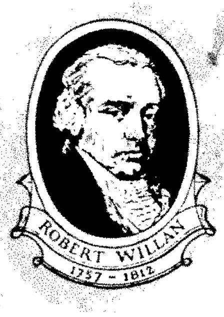

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Department of Dermatology, University of Glasgow G12 8QQ

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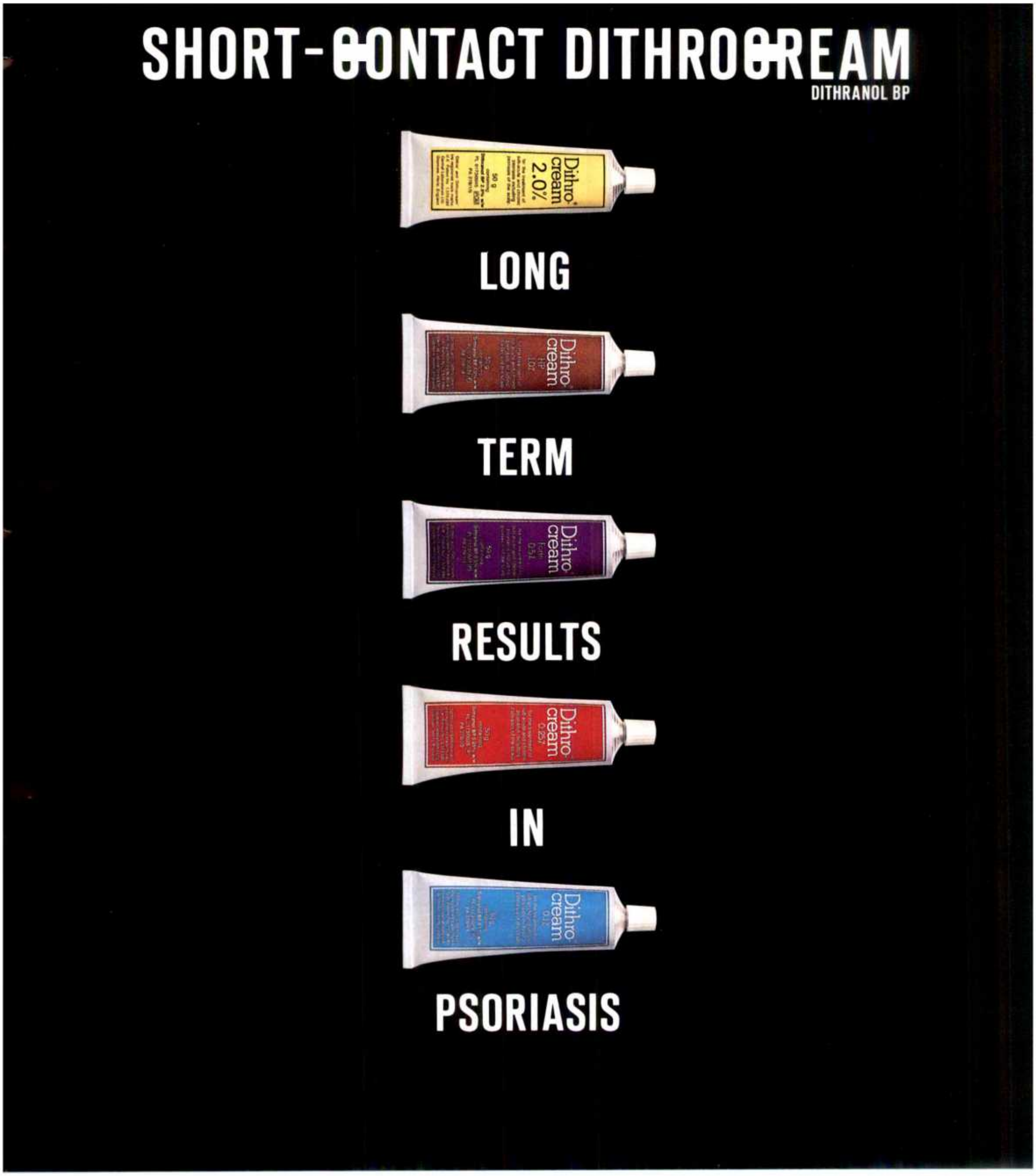

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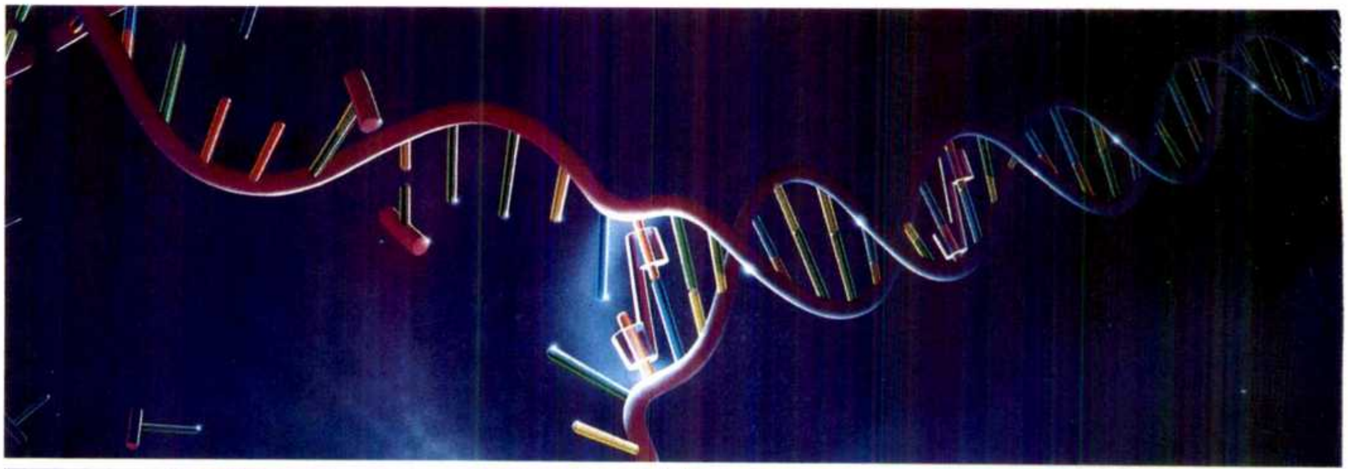

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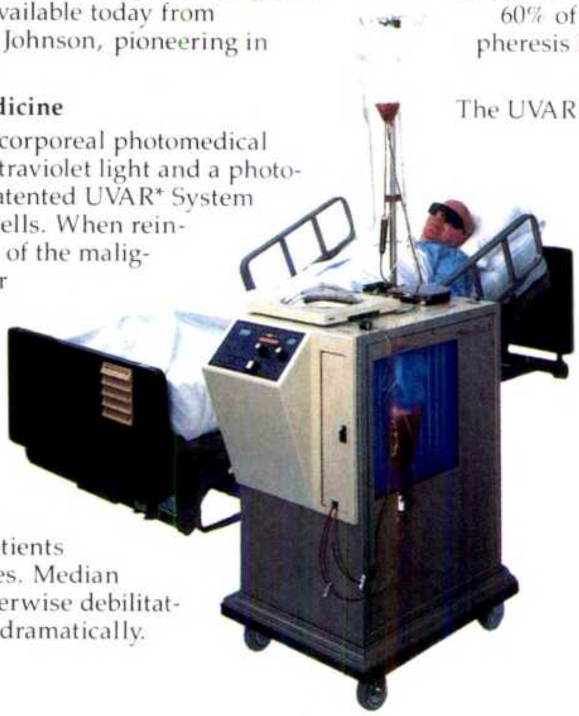

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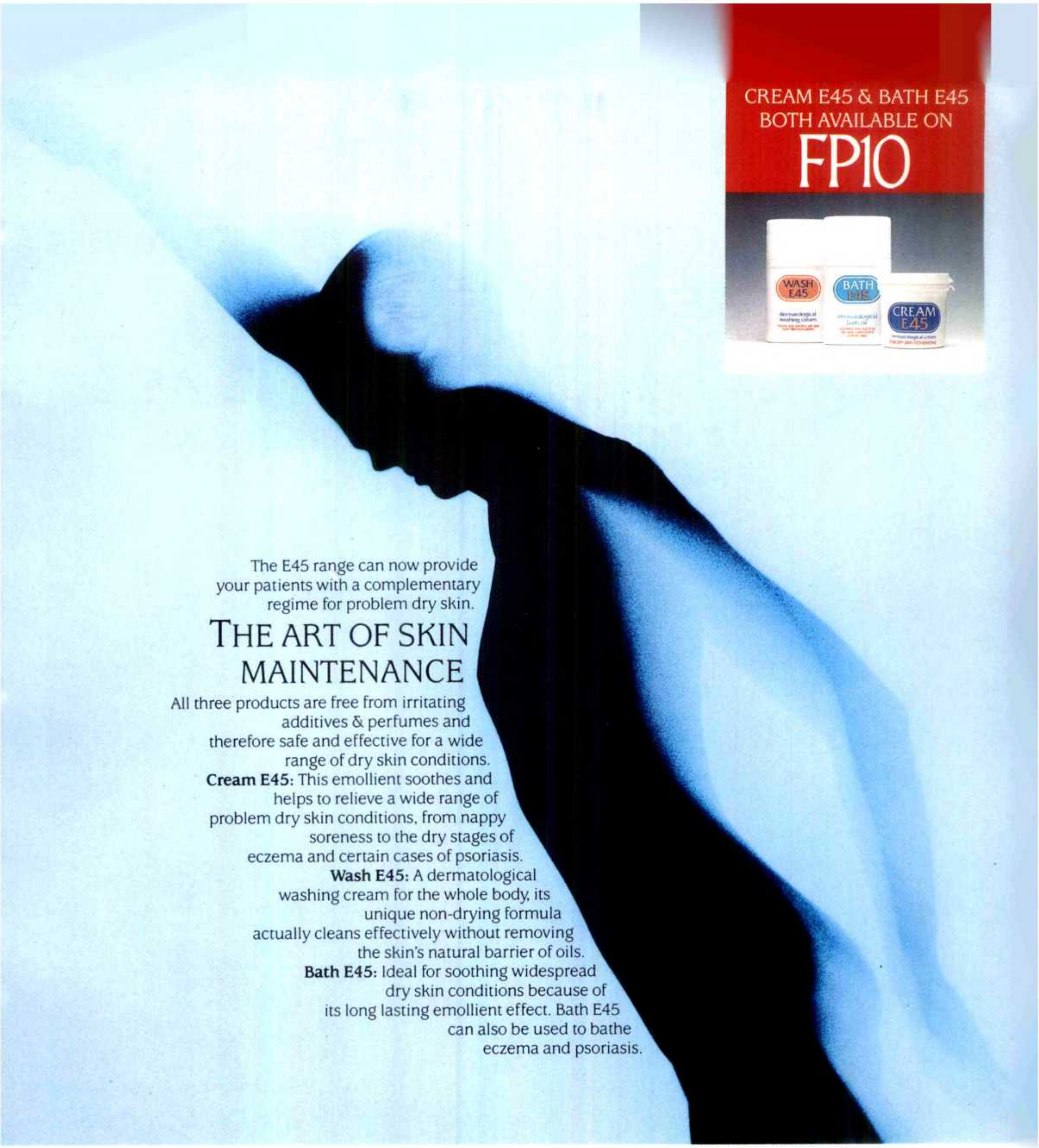

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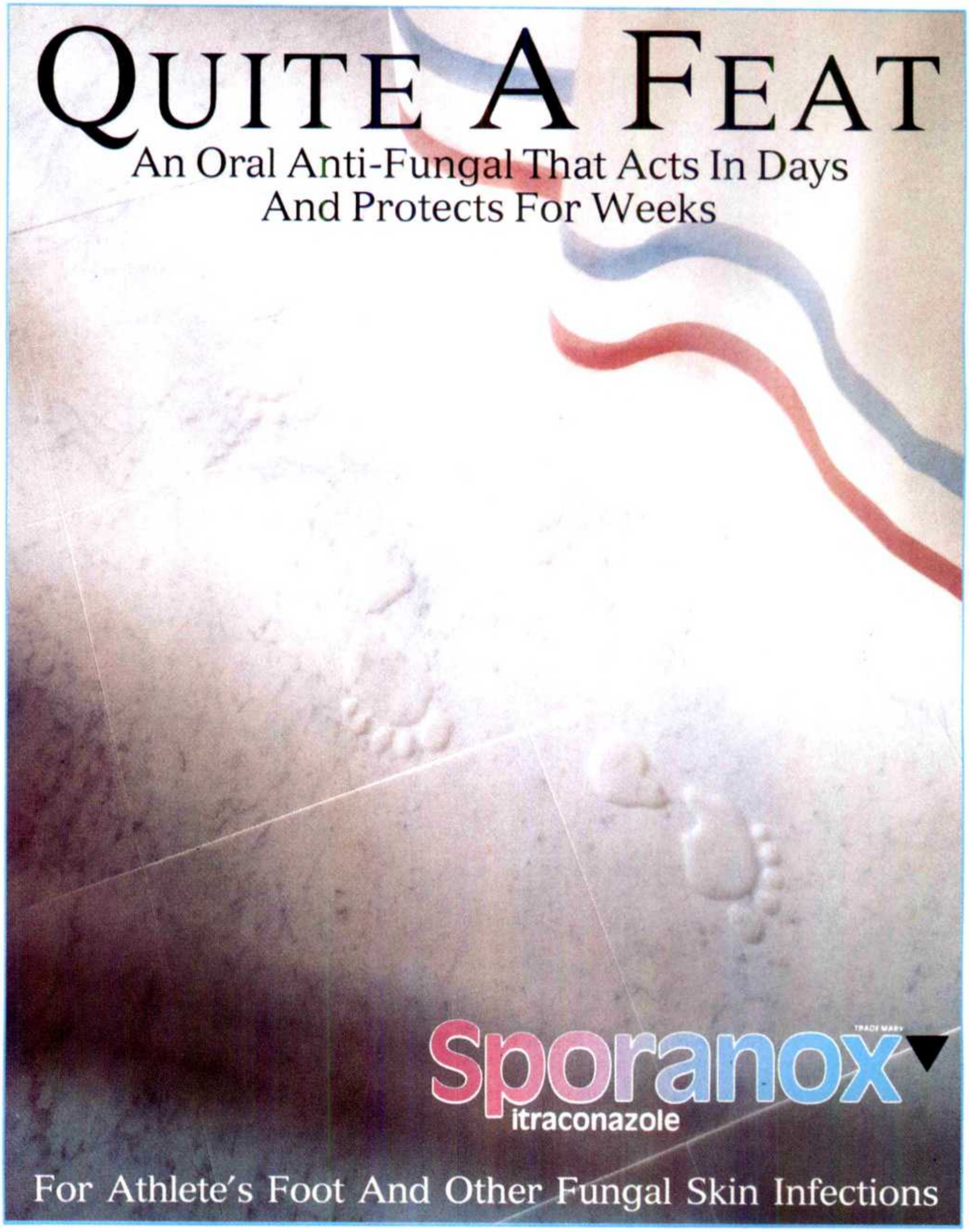
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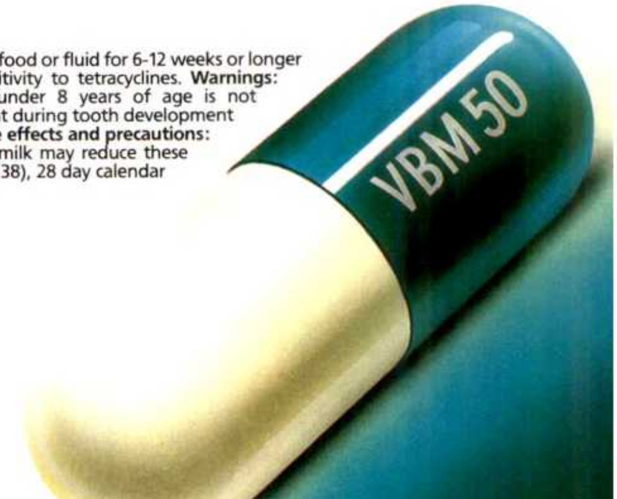

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British Journal of Dermatology (1991) 124, 217–220. ADONIS 000709639100054N

Human monoclonal anti-basement membrane zone antibodies derived from virally transformed lymphocytes of a patient with bullous pemphigoid recognize epitopes associated with hemidesmosomes

H.SHIMIZU*†, T.HASHIMOTO†, T.NISHIKAWA† AND R.A.J.EADY*
*Department of Cell Pathology, Institute of Dermatology, UMDS, University of London, London, UK
†Department of Dermatology, Keio University School of Medicine, Tokyo, Japan

Accepted for publication 25 September 1990

Summary The purpose of this study was to determine the ultrastructural localization of the epitopes recognized by two human monoclonal antibodies designated 5E and 10D, that were derived from virally transformed lymphocytes from a patient with bullous pemphigoid (BP). Previous immunoblotting showed that 5E but not 10D reacted with a 230-kDa protein in extracts of normal human skin. In the present study, indirect immunofluorescence showed that both 5E and 10D bound to the epidermal side of 1 M NaCl-separated normal human skin. Immunoperoxidase electron microscopy showed that 5E and 10D formed discontinuous focal deposits along the basal region of the epidermal basal cells in normal human skin in a similar localization to that seen with whole BP sera. Post-embedding immunogold electron microscopy demonstrated binding of both monoclonal antibodies to the intracellular components of hemidesmosomes. As far as we are aware, 5E and 10D are the first monoclonal antibodies to be described that recognize epitopes of BP antigen associated with hemidesmosomes and should serve as useful probes for future studies.

Direct and indirect immunofluorescence studies have shown that patients with bullous pemphigoid (BP) have *in-vivo* bound and circulating autoantibodies directed against the basement membrane zone (BMZ) of stratified squamous epithelium.[1] Pre-embedding immunoelectron microscopy (EM) has shown that these antibodies bind to antigens associated with the lamina lucida and hemidesmosomes in normal human skin.[2–8] Recent work has indicated that BP antigen is mainly confined to intracellular components of hemidesmosomes but also exists, to a lesser degree, outside the cells.[9,10]

Recently, we established two monoclonal antibodies from virally transformed lymphocytes of a patient with BP.[11] These antibodies, designated 5E and 10D, were found by immunofluorescence to have epitopes in the epidermal BMZ.[11] The purpose of this study was to identify the ultrastructural binding sites of 5E and 10D and to determine whether these monoclonal antibodies recognize epitopes associated with hemidesmosomes or other defined subcellular components of the BMZ.

Methods

Antibodies and sera

5E and 10D human monoclonal antibodies were produced as described previously.[11] Briefly, an enriched fraction of B lymphocytes obtained from the peripheral blood of a patient with BP was incubated with Epstein–Barr virus and then cultured. Supernatants from wells with colony formation were screened for anti-BMZ antibodies by indirect immunofluorescence. The transformants producing anti-BMZ antibodies were cloned and the supernatants were used as monoclonal antibodies. Immunoblotting showed that 5E but not 10D reacts with a 230 kDa protein in the extracts of normal human skin, which is a major component of BP antigen.[12,13] Blocking immunofluorescence experiments disclosed that the epitopes recognized by 5E and 10D are different. Eighteen out of 30 BP sera blocked the reactivity of 5E and 17 blocked 10D, while nine sera

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Correspondence: Professor R.A.J.Eady, Department of Cell Pathology, Institute of Dermatology, St Thomas's Hospital, London SE1 7EH, U.K.

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failed to block either of the two monoclonal antibodies. Sera from three BP patients, including the patient whose peripheral lymphocytes were used for the production of 5E and 10D, as well as three normal human sera were used as controls.

Antigen mapping on 1 M NaCl-separated skin

Using 1 M NaCl-split skin as a substrate,[14] the distribution of the 5E and 10D epitopes was studied by indirect immunofluorescence.

Pre-embedding immunoperoxidase electron microscopy

Using immunoperoxidase as a marker, indirect pre-embedding immuno-EM was performed as previously described.[8] Briefly, 6 μm cryostat sections of normal human skin were incubated in primary antibody followed by incubation with horseradish peroxidase-conjugated rabbit anti-human IgG antiserum (Dakopatts Co., Copenhagen, Denmark). The sections were incubated with diaminobenzidine, fixed in glutaraldehyde and osmium tetroxide, then dehydrated and embedded in epoxy resin using the inverted gelatin-capsule method.[8] Ultrathin sections were cut and stained with uranyl acetate and observed in a JEOL 100 CX transmission-electron microscope.

Post-embedding immunogold electron microscopy

Normal human skin to be used as a substrate for post-embedding immuno-EM was processed as described previously.[9,15] Small pieces (< 1 cm^3) of normal human skin were cryoprotected in 20% glycerol, cryofixed in liquid propane, subjected to freeze substitution in methanol and embedded in Lowicryl K11M (Chemische Werke Lowi, Waldkraiburg, Germany). Ultrathin sections were collected on nickel grids for immunolabelling incubations, all of which were performed at room temperature. The grids were placed on a drop of 1% bovine serum albumin with 5% normal goat serum in phosphate-buffered saline pH 7·4 for 30 min and then incubated in 5E or 10D diluted 1:2 in 0·5% bovine serum albumin with 0·1% gelatin in phosphate-buffered saline (dilution and washing buffer). The sections were further washed in buffer and then exposed to an affinity-purified biotin-conjugated goat anti-human IgG (Sera Lab, Crawley Down, Sussex, U.K.) at a 1:200 dilution for 60 min. The sections were incubated in 1 nm colloidal gold-conjugated streptavidin (Janssen Life Science Products, Olen, Belgium) at a 1:20 dilution for 60 min and finally treated with silver-enhancement solution (Janssen Life Science Products). The sections were stained with 0·2% uranyl acetate, carbon coated, and examined in a transmission-electron microscope.

Controls

Controls for both pre-embedding and post-embedding immuno-EM were performed as described previously.[8,9]

Results

Antigen mapping on 1 M NaCl-separated skin

Both 5E (Fig. 1) and 10D as well as polyclonal antibodies of BP sera bound exclusively to the epidermal side of the separated skin.

Pre-embedding immunoperoxidase EM

Both 5E and 10D showed discontinuous focal deposits along the basal region of the epidermal basal cells (Fig. 2a and b) in a similar immunolocalization to that found with human polyclonal anti-BMZ antibodies of BP sera (data not shown). Normal human sera, used as controls, showed no specific immunolabelling.

Post-embedding immunogold EM

With both 5E and 10D, there was labelling over the intracellular component of hemidesmosomes (Fig. 3a and b). With whole BP sera there was gold labelling of both the intra- and extracellular components of hemidesmosomes as described previously[9] (data not shown).

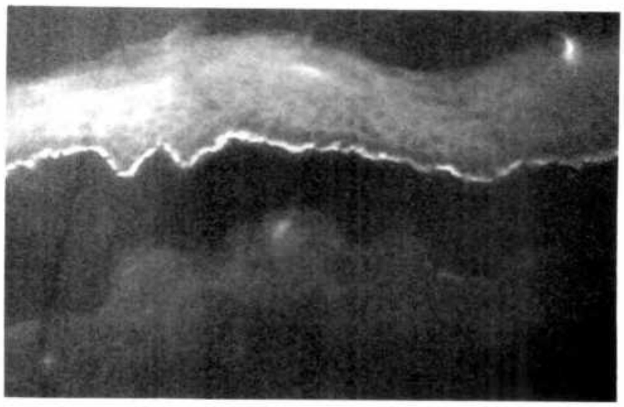

Figure 1. Indirect immunofluorescence showing staining of epidermal side of 1 M NaCl-separated skin using 5E monoclonal antibody. (×250)

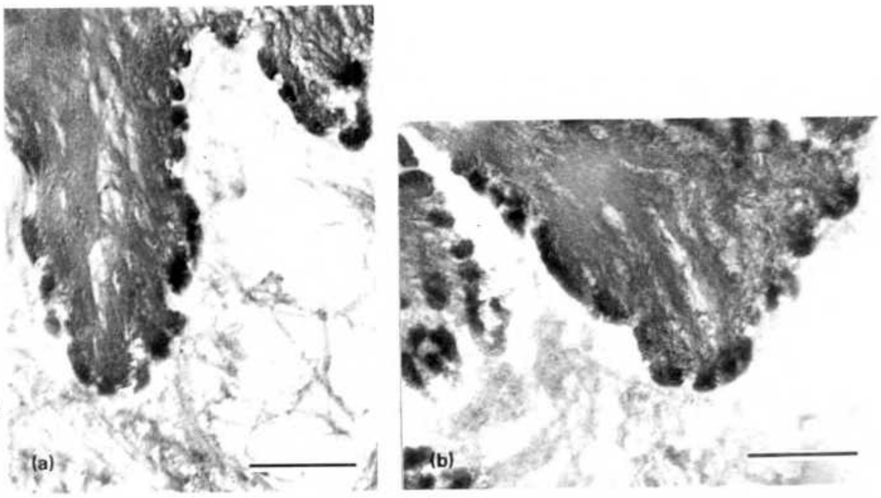

Figure 2. Pre-embedding indirect immunoperoxidase electron microscopy. Reaction products are present as discontinuous focal deposits along the basal region of basal keratinocytes after incubations with both (a) 5E and (b) 10D. Bars = 1 μm.

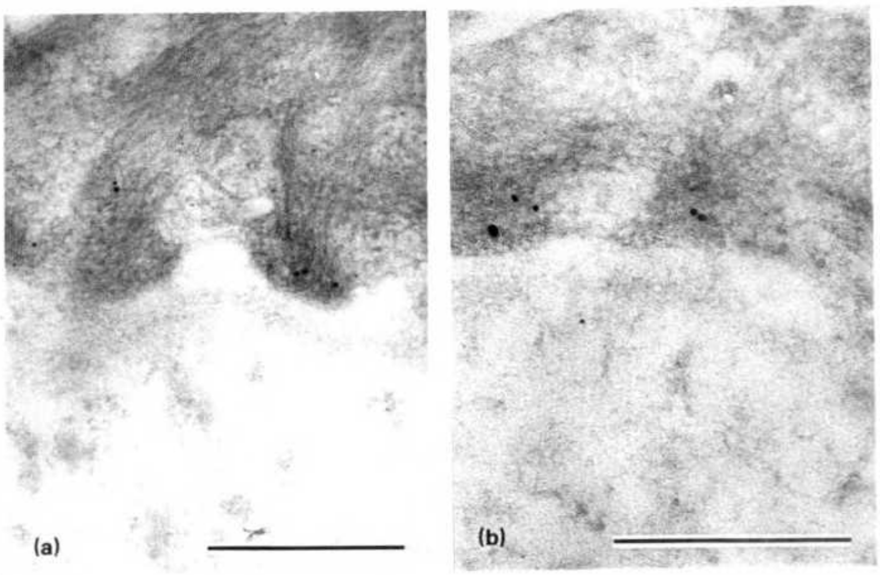

Figure 3. Post-embedding indirect immunogold electron microscopy. With both (a) 5E and (b) 10D there is labelling over the intracellular component of hemidesmosomes. Bars = 0·5 μm.

There was no specific immunolabelling with normal human sera.

Discussion

The results of this study, together with previous blocking immunofluorescence and immunoblotting findings,[11] suggest that both 5E and 10D recognize epitopes of BP antigen associated with hemidesmosomes. Although 10D did not recognize any specific protein by immunoblotting, it did detect the 230-kDa BP antigen by immunoprecipitation (paper in preparation). The blocking immunofluorescence and immunochemical data suggest that 5E and 10D recognize different epitopes of the 230-kDa BP antigen.

Recent investigations employing a post-embedding immuno-EM method similar to that used in the present study, showed that the polyclonal BMZ antibodies of BP sera bound to both the intra- and extracellular components of hemidesmosomes, but predominantly inside the cell,[9] and that a rabbit polyclonal antibody against carboxy terminal epitopes encoded by BP cDNA[16] bound only to the intracellular part of hemidesmosomes.[15] The present study revealed that the epitopes defined by 5E and 10D are localized to the intracellular part of hemidesmosomes.

Several murine monoclonal antibodies against hemidesmosomal components have previously been reported, namely AHS-7,[17] GB3,[18] 19-DEJ-1[19] and 'the hemidesmosomal antibody'.[20] By immunoblotting AHS-7 defines an antigen comprising peptides of various molecular weights ranging from 41 kDa to 160 kDa.[17] GB3 recognizes a very large glycoprotein (apparent molecular weight, 600 kDa) made up of polypeptides in the range of 93·5–150 kDa.[18] 19-DEJ-1 binds to an antigenic epitope in the mid-lamina lucida, exclusively in those areas bordered by overlying hemidesmosomes.[19] A further monoclonal antibody reacts with a 125-kDa polypeptide associated with the hemidesmosomal plaque.[20] None of these monoclonal antibodies has been reported as identifying epitopes associated with BP antigen.

We believe that 5E and 10D are the first monoclonal antibodies to be described that specifically bind to epitopes of BP antigen. They should, therefore, serve as an important tool for future studies on the structure, organization and function of both BP antigen and hemidesmosomes.

Acknowledgments

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Simultaneous assessment of inflammation and epidermal proliferation in psoriatic plaques during long-term treatment with the vitamin D_3 analogue MC903: modulations and interrelations

E.M.G.J.DE JONG AND P.C.M.VAN DE KERKHOF
Department of Dermatology, University of Nijmegen, The Netherlands

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Summary The influence of topical application of MC903, an analogue of 1α,25-dihydroxyvitamin D_3, on psoriatic plaques was investigated during a long-term treatment study. The parameters for epidermal growth and for inflammation were assessed on frozen sections using immunohistochemical methods to elucidate their modulations in time and the interrelations between the different cell types involved during treatment with MC903. Biopsies were taken before and after 1, 2, 4 and 12 weeks of treatment. Monoclonal antibodies against the hyperproliferation-associated keratin 16, against cycling nuclei, and against T lymphocytes, B lymphocytes, Langerhans cells and CD14-positive cells were used in combination with a polyclonal antibody against polymorphonuclear leucocyte (PMN)-elastase.

The earliest change was a statistically significant decrease in PMN after 1 week of treatment followed by a decline of cycling nuclei after 2 weeks. These changes preceded a decrease of T lymphocytes which occurred after 4 weeks. Keratin 16 content tended to diminish after 4 weeks of treatment. $CD14^+$ cells decreased slightly during the observation period, whereas Langerhans cells tended to increase. No B lymphocytes were found. These results suggest that MC903 influences the number of PMN and epidermal growth rather than the number of T lymphocytes.

The observation of a patient with psoriasis and senile osteoporosis who showed clearing of her psoriasis after treatment with 1α,25-dihydroxycholecalciferol,[1] has led to the development of a number of new derivatives of vitamin D_3 which have been used in several trials.[2–4] To minimize the side-effects on blood calcium and phosphate an analogue of 1α,25-dihydroxyvitamin D_3, MC903 (Fig. 1), has been developed which is 100 times less active as a calciotropic agent than 1α,25-dihydroxyvitamin D_3.[5] In clinical trials this analogue has been shown to improve psoriasis.[6–8]

In psoriasis there is both inflammation and epidermal proliferation with an increased recruitment of cycling cells,[9] and the expression of hyperproliferative keratins (keratin 16).[10] Cutaneous inflammation in psoriasis is a complex interplay of polymorphonuclear leucocytes (PMN), T lymphocytes, Langerhans cells and monocytes. The order in which inflammation and proliferation appear during the pathogenesis and disappear during treatment is as yet unknown.

Although the clinical efficacy of MC903 is well established, its mode of action is not yet completely known. *In-vitro* studies have shown that 1α,25-dihydroxyvitamin D_3 has an anti proliferative effect and induces differentiation using a HL60 cell line[11] and keratinocytes.[12,13] Various investigations have also shown an immunomodulating effect of 1α,25-dihydroxyvitamin D_3 including interference with cytokine production and T-cell proliferation,[14–18] these effects also occur with MC903.[19,20]

The aim of the present investigation was to visualize changes in inflammation and epidermal proliferation using immunohistochemical methods during long-term treatment of psoriasis with the vitamin D_3 analogue MC903. The changes observed at different times in the epidermis and dermis in psoriatic plaques were studied.

Correspondence: Dr E.M.G.J.de Jong, Department of Dermatology, University Hospital Nijmegen, Javastraat 104, 6524 MJ Nijmegen, The Netherlands.

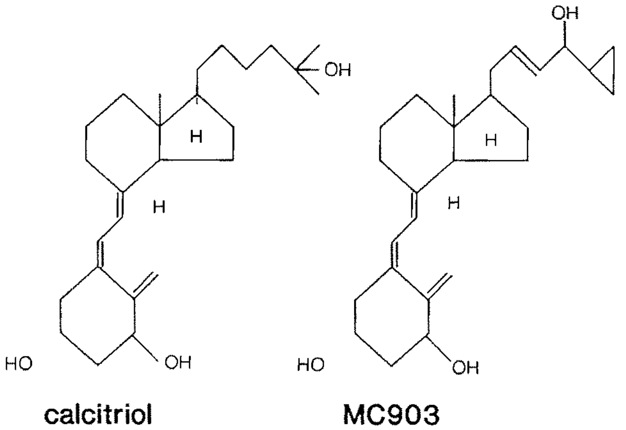

Figure 1. Chemical structure of the vitamin D_3 analogue MC903 and 1α,25-dihydroxycholecalciferol (calcitriol).

Methods

Patients

Eight patients suffering from psoriasis were treated with MC903 ointment (50 μg/g) twice daily (Leo Pharmaceutical Products, Copenhagen, Denmark). These patients had been shown to respond well to MC903-treatment in a previous double-blind in-patient comparison study using MC903 and betamethasone.[21] The patients were included in a multicentre trial covering 12 months of treatment. Their ages ranged between 27 and 85 years, and prior to the study they had received no local treatment for 4 weeks or systemic therapy for their psoriasis for 2 months. The patients had normal values for blood calcium, phosphate, albumin, bilirubin, alkaline phosphatase, aspartate amino transferase, creatinine and urate. The white and red blood cell counts, haemoglobin, platelets and differential counts were all within the normal range. These were measured every 4 weeks. The clinical improvement was assessed using the PASI-score.

Biopsies

Punch biopsies (3-mm diameter) were taken from the psoriatic lesions of six patients before treatment and after 1, 2, 4 and 12 weeks of treatment with MC903. Biopsies were embedded in Tissue Tek OCT compound (Miles Scientific, Naperville, IL, U.S.A.), snap frozen in liquid nitrogen and stored at −80°C. Sections (7 μm) were cut and fixed for 10 min in acetone–ether 60/40 vol% for staining with the antibody Ki-67 and with acetone for the remaining antibodies.

Monoclonal antibodies

To assess epidermal proliferation monoclonal antibodies were used against a nuclear antigen present in the cycling cells (Ki-67, Dakopatts, Copenhagen, Denmark),[22] and against keratin 13 and 16 (Ks8.12, Sigma, St Louis, MO, U.S.A.). Keratin 16 is present in hyperproliferative epidermis,[23] while keratin 13 has not been found in adult human skin.[24] To characterize the inflammatory infiltrate staining was performed with antibodies against leucocyte-elastase which visualizes PMN (Serotec, Oxford, U.K.), T lymphocytes (CD2) (T11, Dakopatts, Copenhagen, Denmark), B lymphocytes (CD22) (pan-B, Dakopatts), CD14-positive cells which represent monocytes and macrophages (WT14, Department of Medicine, Division Nephrology, University Hospital Nijmegen, The Netherlands),[25] and against Langerhans cells (OKT6, Ortho Diagnostic Systems, Raritan, NJ, U.S.A.).[26]

Staining procedures

An indirect peroxidase technique was used for staining with Ks8.12 and Ki-67. Briefly, the slides were incubated for 30 min with the monoclonal antibodies and after washing with phosphate-buffered saline (PBS) were incubated with rabbit anti-mouse immunoglobulin conjugated with peroxidase (RAMPO) for 30 min. A solution of 3-amino-9-ethylcarbazole (AEC) in sodium-acetate buffer pH 4·9 containing 0·01% H_2O_2 was added for 15 min after pre-incubation with sodium acetate buffer pH 4·9. Staining with anti-elastase was done using a direct immunoperoxidase technique. After incubation for 30 min and three washes in PBS, AEC-solution was added following pre-incubation with sodium acetate buffer pH 4·9. Staining with T11, pan-B, WT14 and OKT6 was performed using a peroxidase-anti-peroxidase (PAP) technique. Slides were incubated for 60 min with the monoclonal antibodies. After washing with PBS, an incubation cycle followed of 20 min with rabbit anti-mouse immunoglobulin (RAM-Ig, Dakopatts), two washes with PBS, and 20 min with PAP-complexes (PAP, Dakopatts). This cycle was repeated, then the slides were incubated with sodium acetate buffer pH 4·9 and finally stained in the AEC-solution. All slides were counterstained with Mayer's haematoxylin (Sigma, St Louis, MO, U.S.A.) and mounted in glycerin gelatin.

Histological examinations

Epidermal proliferation was measured by counting the number of Ki-67-positive nuclei per mm length of section, and by assessing Ks8.12 staining in suprabasal and basal layers of the epidermis using a five-point scale: 0, no staining; 1, sporadic staining; 2, minimal staining; 3, moderate staining; 4, pronounced staining.

Dermal inflammation (PMN, T lymphocytes, B lymphocytes, Langerhans cells, CD14-positive cells) was semiquantitatively enumerated by expressing the number of positively stained cells as a percentage of the total number of infiltrate cells:[27] 0, no positive cells; 1, sporadic; 2, 1–25%; 3, 26–50%; 4, 51–75%; 5, 76–99%; 6, 100%. Inflammatory cells in the epidermis were assesed using the five-point scale as used for Ks8.12 staining.

Statistical analysis

For statistical evaluation the Mann–Whitney test was used.

Results

Clinical response

In all patients, MC903 resulted in an improvement of the psoriatic lesions. Total clearance of lesions was not achieved in any of the patients after 12 weeks of treatment. The improvement was most pronounced in the first 4 weeks of treatment ($P<0{\cdot}05$). Continuation beyond this period resulted in only a modest amount of further improvement. No side-effects or flare-ups were observed during treatment.

Epidermal growth

The effect of MC903 on parameters of epidermal growth are summarized in Figures 2–4. The number of actively cycling epidermal cells (Ki-67-positive nuclei) (Figs 2 and 3) showed a statistically significant decrease after 2 weeks of treatment ($P<0{\cdot}03$). After this initial improvement the number of Ki-67-positive nuclei only decreased slightly. Values as found in the uninvolved psoriatic skin by other investigators[28] were not reached in the 12-week observation period. Keratin 16, seen as Ks8.12-staining (Figs 2 and 4), was high in the suprabasal layers of the epidermis. The intensity of this staining remained pronounced and diminished only in the period between 4 and 12 weeks of treatment. However, this decrease was not statistically significant. Basal staining was almost unchanged throughout the whole observation time, and varied only from no staining to sporadic staining in all biopsies. The distribution and intensity of keratin 16 did not switch to the pattern characteristic for clinically uninvolved psoriatic skin which consists of sporadic staining of the basal layer and no staining of the suprabasal compartment.[10]

Inflammation

The density of the inflammatory cells is shown in Figures 5 and 6. In untreated psoriatic plaques PMN, as shown by the antibody anti-elastase, were present as micro-abscesses in the stratum corneum and dispersed in the epidermis. After 1 week of treatment with MC903, only sporadic small micro-abscesses were seen. After 4 weeks the number of PMN had decreased strikingly and no micro-abscesses were found ($P<0{\cdot}01$). Dermal PMN also diminished in this treatment period. Remarkably, after 12 weeks an increase of epidermal as well as in dermal PMN was measured (Fig. 5a). T lymphocytes (T11-positive cells) were numerous in the dermis and epidermis of untreated psoriatic lesions, comprising 76–90% of the dermal infiltrate. Also a considerable epider-

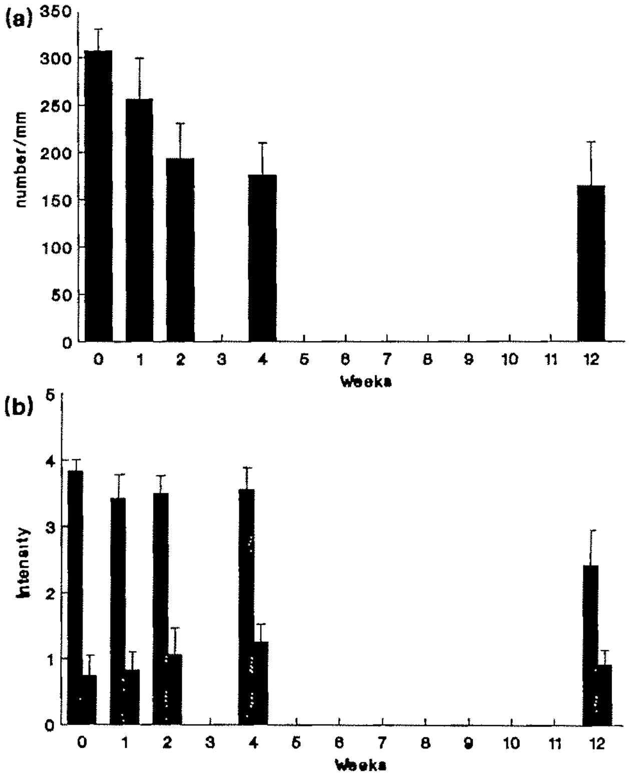

Figure 2. Markers for epidermal growth before and during treatment of psoriatic plaques with MC903, expressed as means±SEM. (a) Ki-67 (cycling epidermal cells) (b) Ks8.12 (epidermal cells expressing keratin 16). ■, suprabasal staining, ■, basal staining.

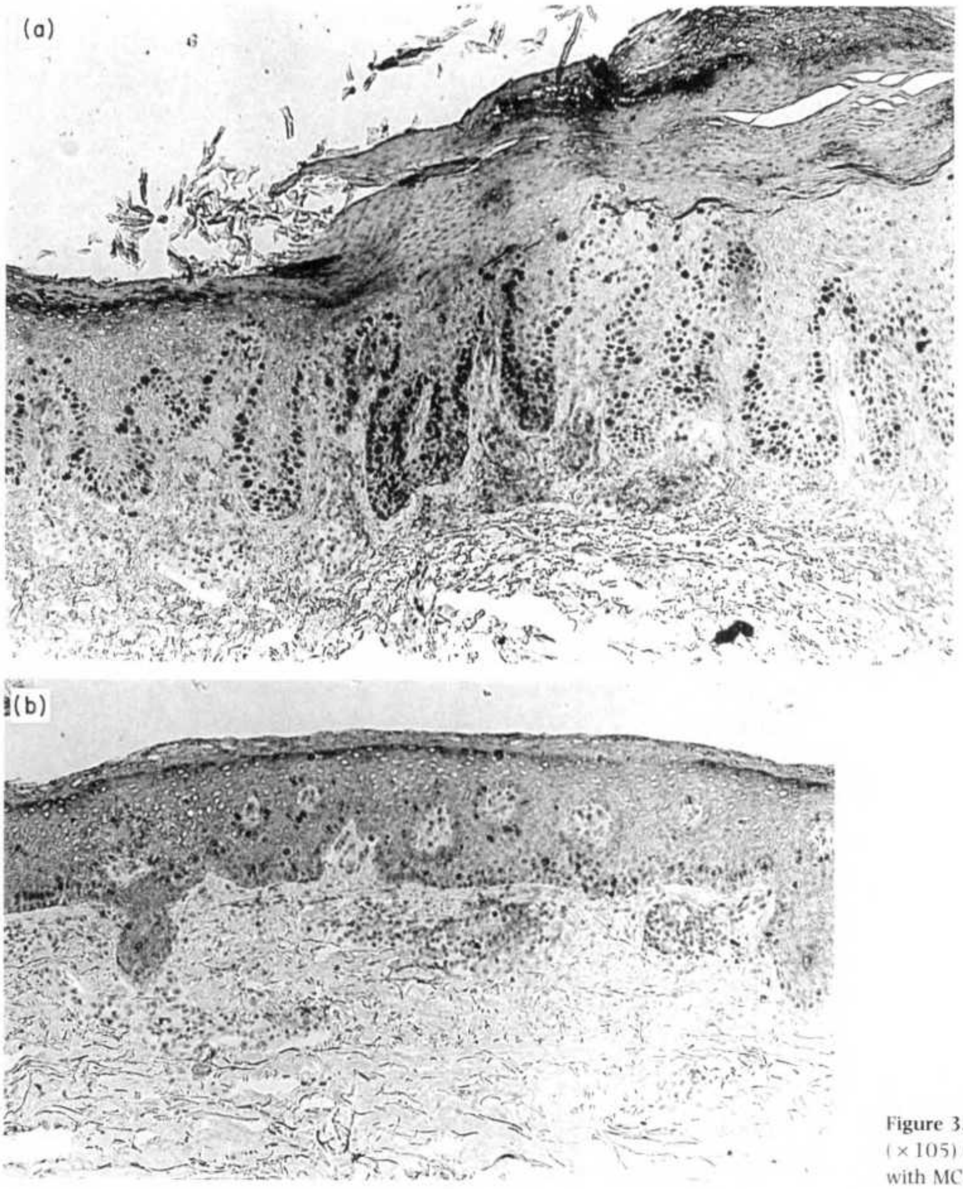

Figure 3. Ki-67 staining (a) before treatment (× 105) (b) after 12 weeks of treatment with MC903 (× 116).

motropism was seen. In the period of treatment with MC903, T lymphocytes diminished gradually in both the dermis and epidermis (Fig. 6), resulting in a borderline significant decrease after 4 weeks ($P=0\cdot05$) (Fig. 5b). B lymphocytes (pan-B-positive cells) were not found in psoriatic skin. CD14-positive cells, assessed by the monoclonal antibody WT14, were found throughout the dermis and epidermis. Their numbers decreased slightly but this was not statistically significant (Fig. 5c) and their distribution pattern did not show a consistent change. Epidermal Langerhans cells, visualized by OKT6, tended to increase within the first week ($P>0\cdot05$) of treatment and then remain at the same level (Fig. 5d). The pattern of distribution of these cells correlated with the general histological appearance. Pretreatment biopsies, showing marked acanthosis, papillomatosis and parakeratosis, demonstrated focal aggregates of Langerhans cells intermingled with Langerhans cell-free zones, whereas biopsies of treated skin with a more modest degree of involvement, showed a more diffuse network of Langerhans cells. Dermal Langerhans cells decreased slightly during treatment with MC903.

Relation between clinical and histological scores

The decrease of the number of epidermal PMN and the

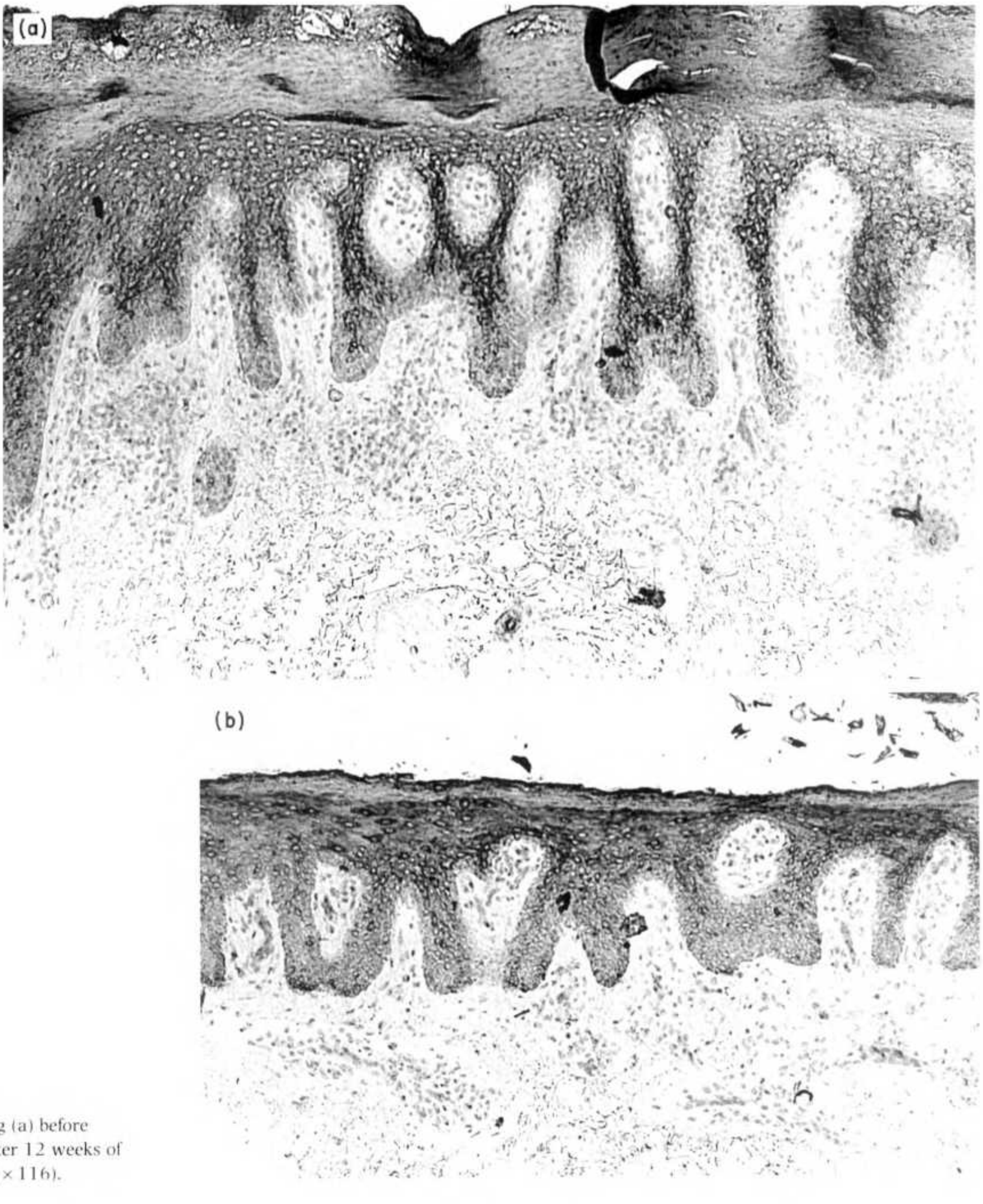

Figure 4. Ks8.12 staining (a) before treatment (× 105) (b) after 12 weeks of treatment with MC903 (× 116).

number of Ki-67-positive nuclei preceded the decrease in PASI score. T lymphocytes diminished simultaneously with the clinical scores. Also Ks8.12 binding tended to diminish at the same time as the PASI score.

The patient group was divided into a group showing marked improvement (PASI after 12 weeks <3, $n=4$) and a group showing slight to moderate improvement (PASI after 12 weeks >3, $n=2$). The decrease in Ki-67-positive nuclei was twice as high in the group showing marked improvement compared to the group showing slight to moderate improvement (decrease in stained nuclei/mm length of section: 157 and 70, respectively). The reduction of epidermal PMN in the group showing marked improvement was also twice as high as that in the other group (reduction of 2·25 and 1, respectively). The number of T lymphocytes was substantially reduced in the first group, whereas the decrease in the second group was marginal (reduction of 1·4 and 0·1, respect-

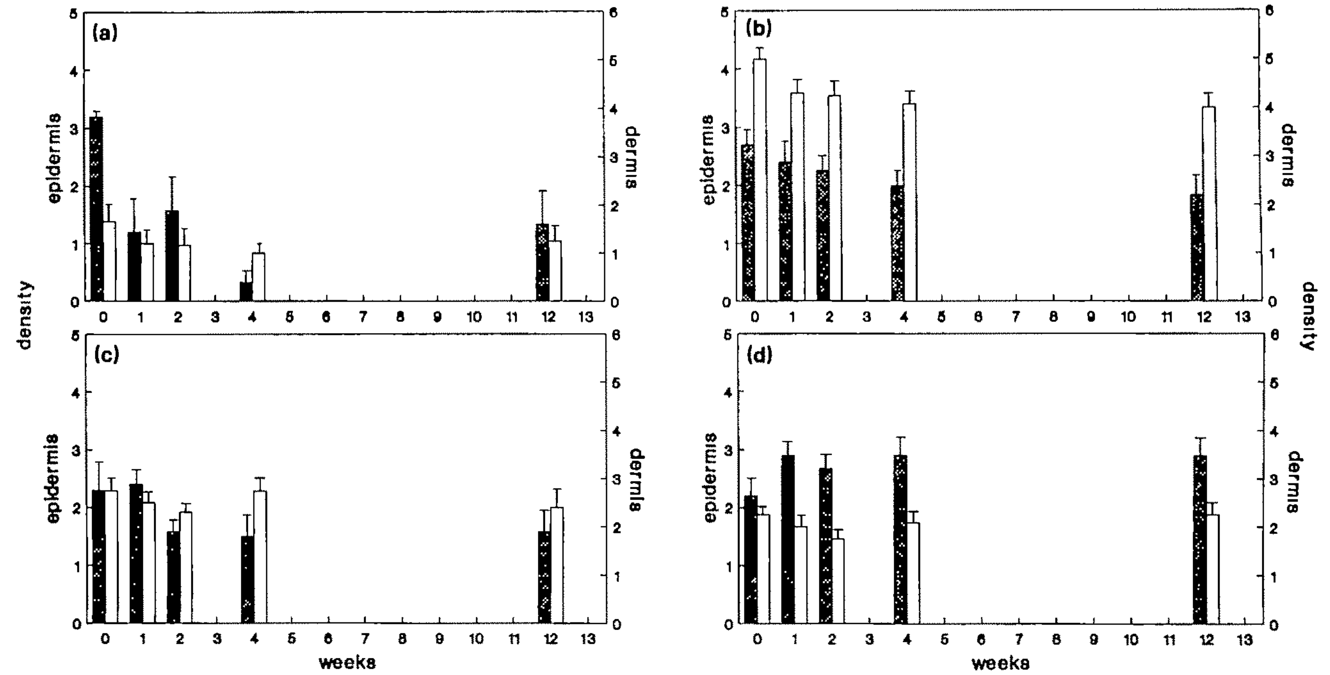

Figure 5. Markers of inflammation before and during treatment of psoriatic plaques with MC903, expressed as means ± SEM. (a) Anti-elastase (PMN), (b) T11 (T lymphocytes), (c) WT14 (CD14-positive cells), (d) OKT6 (Langerhans cells). ■, epidermal cells; □, dermal cells.

ively). Ks8.12 binding diminished in both groups to the same extent (2·4 and 2·8, respectively).

Discussion

This study investigated the histological change found in psoriatic lesions during treatment with MC903. The earliest changes was a pronounced decrease in the number of PMN, which was statistically significant after only 1 week of treatment ($P<0{\cdot}05$). At the same time the number of Langerhans cells tended to increase, but this was not significant. After 2 weeks there was a significant decrease in Ki-67-positive nuclei of almost 40% ($P<0{\cdot}05$). The PASI scores were reduced after 4 weeks of treatment. There was a reduction in the number of T lymphocytes approaching significance ($P=0{\cdot}05$). Ks8.12 staining diminished only in the period between 4 and 12 weeks of treatment. However, the number of PMN during this period showed a slight increase ($P>0{\cdot}05$). The group of patients with the lowest PASI scores after 12 weeks of treatment showed the most marked reduction in the number of Ki-67-positive nuclei, epidermal PMN and T lymphocytes.

The skin is not only the site where vitamin D is produced,[29] but keratinocytes also have receptors for 1α,25-dihydroxyvitamin D_3 and function as a target organ.[30] There is growing evidence that 1α,25-dihydroxyvitamin D_3 can suppress the proliferation and induce differentiation of different cell types including keratinocytes,[11-13] although it is not clear how this is achieved. 1α,25-dihydroxyvitamin D_3 possesses a cytosolic receptor[31] that is transported to the nucleus after binding, and is thought to work through DNA transcription.[32] In addition, several authors reported a rapid effect of 1α,25-dihydroxyvitamin D_3 on intracellular calcium transport[33] and on cGMP in human skin fibroblasts.[34] These observations suggest that more immediate mechanisms, not mediated via the genome, might be of relevance. MC903 is very similar to the natural metabolite 1α,25-dihydroxyvitamin D_3; both bind to the same receptor, induce differentiation and inhibit cell proliferation at the same concentrations *in vitro*. In addition, MC903 is at least 100 times less potent in its effect on calcium metabolism *in vivo*.[5]

In the present investigation a rapid decrease of the number of proliferating cells, measured as Ki-67-positive nuclei, was found after 2 weeks, resulting in a total decrease of 48% after 12 weeks. Thus, the *in-vitro* effect on proliferation of MC903 is also found in *in vivo*. These findings agree with values found by de Mare *et al.* who observed a decrease in DNA content after 6 weeks of treatment with MC903 using flow cytometry. This effect

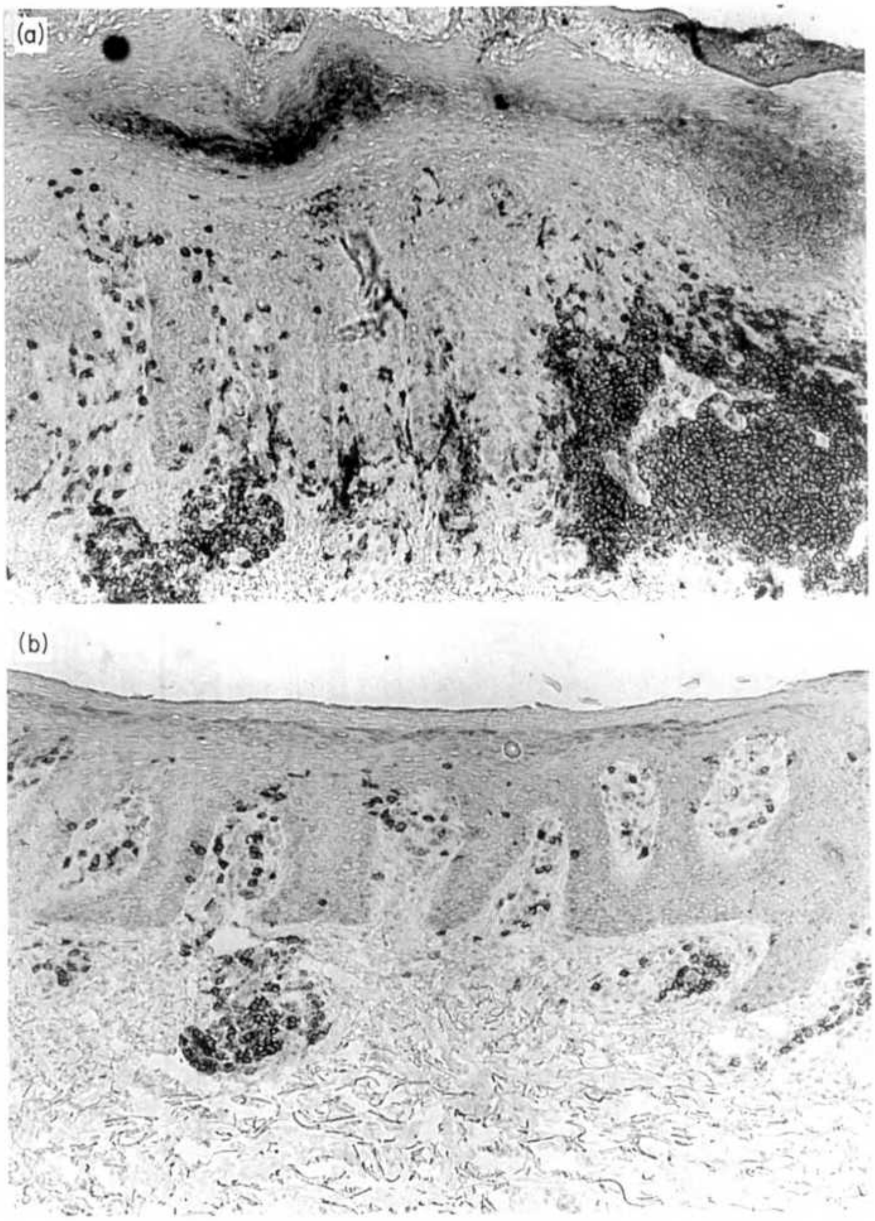

Figure 6. T11 staining (a) before treatment, (b) after 4 weeks of treatment with MC903 (× 136).

was comparable to that of betamethasone.[21] Ks8.12 binding, showing expression of keratin 16, showed a tendency to diminish only after 4–12 weeks of treatment. In a previous investigation, using flow cytometry, Ks8.12 binding proved to be reduced significantly after 6 weeks of treatment with MC903.[21] This discrepancy can be explained by the precise data provided by this approach. Although the number of cycling cells is reduced at an early stage of treatment with MC903, the reduction of the intraepidermal accumulation of PMN preceded the antiproliferative effect *in vivo*.

Receptors for 1α,25-dihydroxyvitamin D_3 are present in cell types involved in immunological reactions, such as activated T lymphocytes, B lymphocytes, monocytes and thymocytes.[15] Production of cytokines such as IL-1, IL-2, IL-6 and EGF-β, is modulated by 1α,25-dihydroxyvitamin D_3 and MC903.[14,16–20] It has been suggested that IL-1 might induce epidermal proliferation.[35] The present study demonstrates the effect of MC903 on the immune system *in vivo*. At first, the accumulation of PMN diminishes and after the most significant reduction of epidermal proliferation at 2 weeks the number of T lymphocytes diminishes by 4 weeks. CD14 positive cells tend to be slightly reduced during the 12-week treatment with MC903. The reappearance of PMN and the slight reduction of the remaining parameters during 4–12 weeks of treatment is intriguing and further studies to explain these findings are required. The molecular basis of the interference of MC903 with inflammation *in vivo* remains speculative. However, based on *in-vitro* observations, it is feasible that MC903 works via modulation of production of IL-1 and IL-2 or by interference with the response of keratinocytes to interleukin 1.

Well known antipsoriatic therapies such as UV-irradiation and corticosteroids[36] decrease epidermal Langerhans cells during treatment. In this study the number of Langerhans cells appeared to increase during therapy with MC903. A reduction of the number of Langerhans cells does not seem to be an indispensable condition for improvement.

The present study suggests that the effect of the vitamin D_3 analogue MC903 is not primarily via interference with the number of T lymphocytes. MC903 might alter the response of keratinocytes to interleukin 1, resulting in a down-regulation of epidermal proliferation. The PMN and epidermal growth seem to be targets with substantial relevance.

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$1\alpha,25(OH)_2$ vitamin D_3 increases intracellular calcium in human keratinocytes

B.BITTINER, S.S.BLEEHEN AND SHEILA MACNEIL*

Department of Dermatology, Royal Hallamshire Hospital, Sheffield S10 2JF, U K.
**Department of Medicine, Clinical Sciences Centre, Northern General Hospital, Sheffield S5 7AU, U.K.*

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Summary

Vitamin D_3 metabolites have been found to improve psoriasis but their mechanism of action is not clear. Keratinocyte proliferation and differentiation are known to be dependent on calcium concentrations *in vitro*. The aim of this study was to examine whether $1\alpha,25(OH)_2$ vitamin D_3 had any direct effect on intracellular free calcium concentrations in cultured keratinocytes. A response to $1\alpha,25(OH)_2$ vitamin D_3 was seen in 88% of monolayers of normal human keratinocytes attached to glass coverslips. An increase in intracellular free calcium was seen in 80% of the reactive cultures, with over half the responses occurring within 30 s of exposure to $1\alpha,25(OH)_2$ vitamin D_3 and the remainder occurring within minutes. Responses could be seen at physiological concentrations of $1\alpha,25(OH)_2$ vitamin D_3 and were not blocked by the protein synthesis inhibitor cycloheximide. The response to $1\alpha,25(OH)_2$ vitamin D_3 took the form of rapid transient increases in intracellular free calcium in 29 out of 59 coverslips. The basal intracellular free calcium was calculated to be 245 ± 47 nM rising to a maximum of 834 ± 267 nM (mean ± SEM; $n = 20$) following exposure to $1\alpha,25(OH)_2$ vitamin D_3. We conclude that $1\alpha,25(OH)_2$ vitamin D_3 acts directly on keratinocytes to increase intracellular free calcium and that this may be relevant to its mechanism of action in psoriasis.

Attention has recently been focused on the relationship between vitamin D_3 metabolism and the skin following the finding that vitamin D_3 metabolites can improve psoriasis.[1–4] It has been known for many years that vitamin D_3 is formed in the skin on exposure to ultraviolet light. Subsequent hydroxylation of vitamin D_3 in the liver and kidney leads to the formation of the active metabolite $1\alpha,25(OH)_2$ vitamin D_3.[5] Towards the end of the 1970s keratinocytes were found to possess nuclear receptors for $1\alpha,25(OH)_2$ vitamin D_3[6] but several more years elapsed before the skin was widely regarded as a target tissue for this hormone.[7] More recently it has been shown that human keratinocytes are capable of synthesizing $1\alpha,25(OH)_2$ vitamin D_3 *in vitro* from 25OH vitamin D_3, the major circulating form of vitamin D_3 *in vivo*.[8,9]

Vitamin D was used systemically as a therapy for psoriasis as long ago as the 1930s but interest waned because of its toxicity.[5] Interest was rekindled by a case report of a patient whose psoriasis dramatically cleared when treated with $1\alpha(OH)$ vitamin D_3 for senile osteoporosis. Subsequently, open and controlled trials of $1\alpha,25(OH)_2$ vitamin D_3 and other vitamin D_3 analogues have demonstrated benefit in patients with psoriasis.[2–4]

In vitro studies have shown that $1\alpha,25(OH)_2$ vitamin D_3 reduces proliferation and promotes differentiation in cultured keratinocytes.[10] This effect has been demonstrated at physiological concentrations of $1\alpha,25(OH)_2$ vitamin D_3 and also with analogues including the non-calciotropic MC 903.[11] Similar effects of $1\alpha,25(OH)_2$ vitamin D_3 have been found in other cell types[5] suggesting that the action is mediated through a fundamental, but as yet undetermined, cell process.

An effect of $1\alpha,25(OH)_2$ vitamin D_3 on intracellular calcium could be relevant to the response of cultured keratinocytes to this hormone *in vitro*. $1\alpha,25(OH)_2$ vitamin D_3 has recently been shown to increase the intracellular calcium in other cell types, such as murine B16 melanoma cells[12] and hepatocytes.[13] As in all cells, calcium plays an important role in keratinocyte proliferation and differentiation. When keratinocytes are cultured in low-calcium media the cells show accelerated proliferation but do not fully differentiate or form desmosomes.[14] However, the cells revert to normal on restoration of the extracellular calcium to physiological concentrations.[15] Thus, the changes induced by increasing extracellular calcium from low to normal are similar to those found by the addition of physiological amounts of $1\alpha,25(OH)_2$ vitamin D_3 to cultured keratinocytes. This

Correspondence: Dr B.Bittner.

raises the possibility that the effects of 1α,25(OH)$_2$ vitamin D$_3$ may be mediated by changes in calcium concentration within the cell. Although 1α,25(OH)$_2$ vitamin D$_3$ has been shown to increase free intracellular calcium in other cell types[12,13] there has been no equivalent study in human keratinocytes. The aim of this study was to examine the effects of physiological concentrations of 1α,25(OH)$_2$ vitamin D$_3$ on the free intracellular calcium concentration in human keratinocytes to improve our understanding both of 1α,25(OH)$_2$ vitamin D$_3$ biology and, in particular, of the mechanism of action of this hormone in the treatment of psoriasis.

Methods

The reagents used in this series of experiments were obtained from the following sources: Dulbecco's modified Eagles' medium (DMEM) and fungizone from Flow, Ayrshire, U.K.; trypsin/EDTA, penicillin/streptomycin and glutamine from Imperial Laboratories Ltd., Andover, U.K.; digitonin, cycloheximide, epidermal growth factor (EGF) and bombesin from Sigma Chemical Company, Poole, Dorset, U.K.; Fura 2, Fura 2-AM and ionomycin from Calbiochem, Behring Diagnostic Laboratories, CA, U.S.A.; dispase from Boehringer Corporation (London) Ltd.; vitamin D$_3$ and 1α,25(OH)$_2$ vitamin D$_3$ were kind gifts from Shiseido Ltd., Tokyo, Japan. The spectrofluorimeter was a Kontron SFM25 obtained from Kontron Instruments Ltd., Watford, Herts, U.K.

Human keratinocyte cultures

Human keratinocytes were obtained from neonatal foreskins. The subcutaneous tissue was removed and the foreskins were cut into small pieces and incubated with 0·1% dispase for 24–48 h at 4°C. The separated epidermal sheets were incubated in trypsin 0·25%/EDTA 0·02% for 10 min at 37°C. The cells obtained were plated onto glass coverslips coated with rat tail collagen at a density of $2{\cdot}5\times10^5$/cm^2 and were cultured in DMEM with 10% FBS, penicillin 100 IU/ml, streptomycin 100 μg/ml, fungizone 2·5 μg/ml and glutamine 2 mM. The coverslips were used to estimate free intracellular calcium once the cells had reached confluence with early colony formation, approximately 4 days after seeding.

Intracellular calcium estimations

The free intracellular calcium concentrations of the keratinocytes were estimated using the fluorochrome Fura 2-AM. The cells were loaded at room temperature for 45 min in balanced salt solution (BSS) containing 135 mM NaCl, 4·5 mM KCl, 1·5 mM CaCl$_2$, 0·5 mM MgCl$_2$, 5·6 mM glucose and 10 mM Hepes, pH 7·4 to which 4 μM Fura 2-AM was added. The coverslips were washed twice with BSS to remove excess Fura 2-AM and were placed across the diagonal of a 1-cm quartz cuvette, supported 0·5 cm above the base of the cuvette to allow the addition of a plastic coated magnetic stirrer bar to the cuvette. Cells on coverslips incubated in BSS faced the excitation beam which had an angle of incidence of 45° to the keratinocytes. The cuvette contents were continuously stirred with a magnetic stirrer. Experiments were conducted at an excitation wavelength of 340 nm and emissions collected at 490 nm.

Traces were calibrated at the end of the experiment by the following method. The maximum fluorescence of the dye (F$_{max}$) was obtained by adding either digitonin (10 μM) or the calcium ionophore ionomycin (10 μM) which permits saturation of the dye by allowing calcium to enter the cell. In our system both digitonin and ionomycin gave similar values for F$_{max}$. The Fura 2 was then quenched using 100 μM MnCl$_2$ and F$_{Mn}$ obtained. The fluorescence of the dye in the absence of calcium (F$_{min}$) can then be calculated from the equation:

$$F_{min} = F_{Mn} + \frac{(F_{max} - F_{Mn})}{3}$$

which is based on the same equation derived for Quin 2.[16] The intracellular free calcium is derived from the formula

$$[Ca] = Kd\frac{[F - F_{min}]}{[F_{max} - F]}$$

where Kd is the dissociation constant of Fura 2 (224 nM) and F is the measured fluorescence in arbitrary units.

Several additions of vehicle (BSS 30 μl) were made as controls before the additions of active agents in BSS. Control additions of BSS alone were also made intermittently throughout the experiments.

Results

A total of 67 coverslips were examined and a change in intracellular free calcium in response to 1α,25(OH)$_2$ vitamin D$_3$ was obtained in 59 (88%). The response of keratinocytes to 1α,25(OH)$_2$ vitamin D$_3$ was variable; the types of response elicited are shown in Figure 1. Figure 1(a) shows an immediate small increase in intracellular calcium concentration with increased baseline noise in response to 10^{-7} M 1α,25(OH)$_2$ vitamin D$_3$. There was no further response to extra additions of

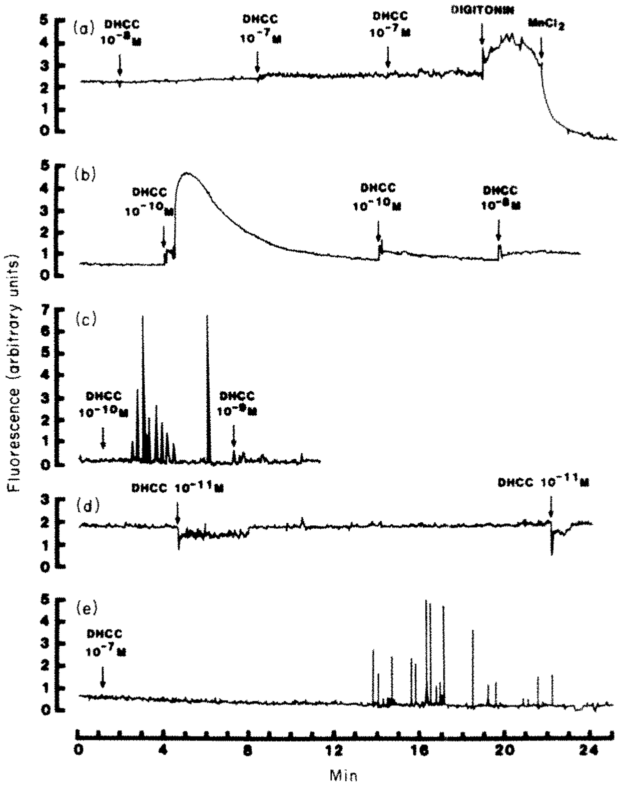

Figure 1. Examples of the variation in response to $1\alpha,25(OH)_2$ vitamin D_3 (DHCC) seen in individual coverslips of human keratinocytes. Cells were cultured as detailed in Methods and exposed to increasing concentrations of $1\alpha,25(OH)_2$ vitamin D_3. (a)-(e) show responses in individual coverslips.

hormone. The trace also demonstrates the response of keratinocytes to 10 μM digitonin followed by 100 μM manganese chloride from which the experiment was calibrated. Figure 1(b) shows an immediate large increase in free intracellular calcium in response to 10^{-10} M $1\alpha,25(OH)_2$ vitamin D_3, which fell to resting levels over a period of several minutes. Further responses to 10^{-10} M and 10^{-8} M $1\alpha,25(OH)_2$ vitamin D_3 were markedly attenuated. Figure 1(c) shows a burst of high frequency spikes occurring approximately 90 s after the addition of 10^{-10} M $1\alpha,25(OH)_2$ vitamin D_3 which lasted for 3 min. This type of spiking response was elicited in 29 of 59 responsive coverslips. Figure 1(d) shows an immediate small reduction in free intracellular calcium with an increase in baseline noise after the addition of 10^{-11} M $1\alpha,25(OH)_2$ vitamin D_3. Occasionally the response of the keratinocytes was delayed for several minutes, an example of which is shown in Figure 1(e). Here a 12 min delay in the response to 10^{-7} M $1\alpha,25(OH)_2$ vitamin D_3 was followed by spiking which lasted for approximately 10 min.

The types of response to $1\alpha,25(OH)_2$ vitamin D_3 are summarized in Figure 2. There was in increase in free intracellular calcium concentration (whether immediate or delayed) in 47 of 59 coverslips (80%), with an immediate increase in baseline noise (8%), being the next most common response. The variability in responses was noted even in replicate cultures from the same donor.

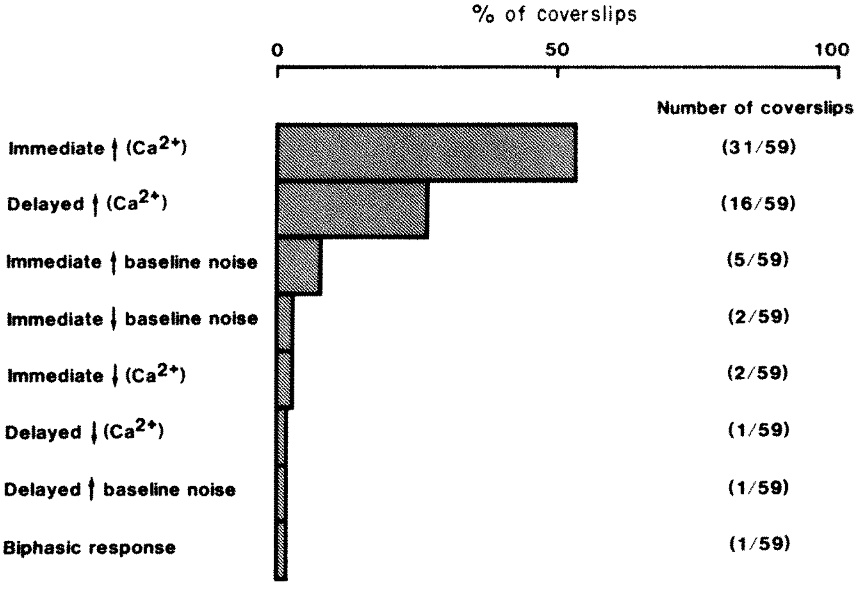

Figure 2. Analysis of the type of responses seen to $1\alpha,25(OH)_2$ vitamin D_3: 59 coverslips which showed some response to $1\alpha,25(OH)_2$ vitamin D_3 were assigned to one of the descriptive categories of response. Any response occurring within 30 s was considered to be immediate. The results are depicted as a histogram of the % frequency of response seen.

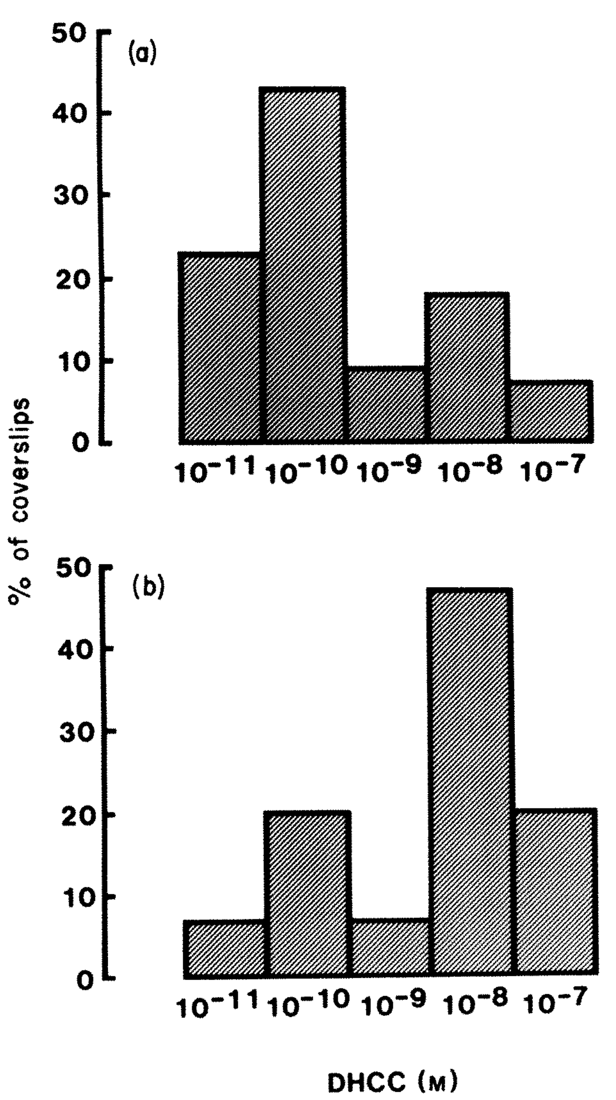

Figure 3. Sensitivity of keratinocytes to 1α,25(OH)$_2$ vitamin D$_3$ (DHCC). Coverslips were exposed to progressively increasing concentrations of 1α,25(OH)$_2$ vitamin D$_3$. The lowest concentration of 1α,25(OH)$_2$ vitamin D$_3$ producing a change in intracellular free calcium was noted and expressed as a percentage. Coverslips were subdivided into those which were exposed to 1α,25(OH)$_2$ vitamin D$_3$ (a) first or (b) after exposure to other agents.

The sensitivity of the keratinocytes to 1α,25(OH)$_2$ vitamin D$_3$ varied depending on whether it was the first agent used or if it was added after other agonists (Fig. 3). The other agonists were epidermal growth factor, foetal bovine serum and bombesin. When 1α,25(OH)$_2$ vitamin D$_3$ was the first agonist used 44 of 48 coverslips (92%) responded and 33 of the 44 responses (75%) occurred at physiological concentrations (10^{-11}–10^{-9} M). When 1α,25(OH)$_2$ vitamin D$_3$ was added as a second agonist a response was obtained in 15 of 19 coverslips (79%), but a supraphysiological concentration (10^{-8}–10^{-7} M) was required to elicit the response in 11 of these 15 (73%).

For various reasons it was only feasible to quantify the free intracellular calcium concentration in 20 of the 59 responsive coverslips. Where this was possible the basal calcium was calculated to be 245 ± 47 nM rising to 834 ± 267 nM (mean ± SEM; $n = 20$) after the addition of physiological concentrations of 1α,25(OH)$_2$ vitamin D$_3$.

A total of eight coverslips were examined following incubation for 24 h with 10^{-4} M cycloheximide in order to block protein synthesis. Seven of the eight (87%) responded to 1α,25(OH)$_2$ vitamin D$_3$ and five of the seven responding (73%) did so at physiological concentrations (10^{-10} M–10^{-9} M), results similar to those obtained in the absence of cycloheximide. Figure 4(a) shows a response to 10^{-10} M 1α,25(OH)$_2$ vitamin D$_3$ in cells pretreated with cycloheximide.

Preliminary data has shown that keratinocytes were also able to respond to vitamin D$_3$ itself. An example is shown in Figure 4 where the responses to vitamin D$_3$ (Fig. 4b) and 1α,25(OH)$_2$ vitamin D$_3$ (Fig. 4c) are compared in the same coverslip. A total of six experi-

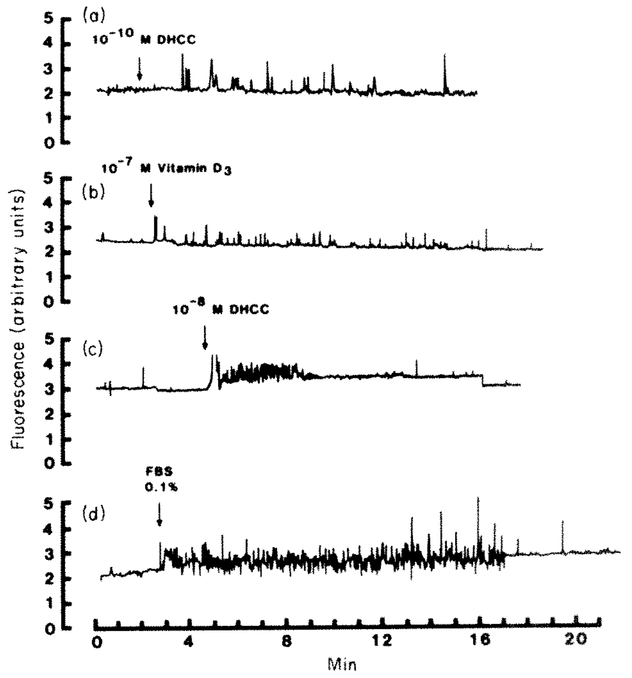

Figure 4. Effect of cycloheximide, vitamin D$_3$ and foetal bovine serum on intracellular free calcium in human keratinocytes. (a) Example of a response to 10^{-10} M 1α,25(OH)$_2$ vitamin D$_3$ (DHCC) in a coverslip of keratinocytes pretreated for 24 h with 10^{-4} M cycloheximide. (b) and (c) Coverslip of cells responding to vitamin D$_3$ and subsequently to 1α,25(OH)$_2$ vitamin D$_3$. (d) Response of a coverslip of cells to the addition of 0·1% foetal bovine serum.

ments were performed with vitamin D_3 and a response was elicited in four at concentrations of 10^{-11}, 10^{-8}, 10^{-7} and 10^{-7} M vitamin D_3.

When foetal bovine serum was used as an agonist the usual response was that of immediate and frequent oscillations in intracellular calcium (Fig. 4d).

Discussion

In this study we have demonstrated for the first time that 1α,25(OH)$_2$ vitamin D_3 can acutely increase intracellular free calcium in normal human keratinocytes. The ability of skin cells to respond to this hormone in this way throws new light on vitamin D_3 biology and possibly on the mechanism of action of 1α,25(OH)$_2$ vitamin D_3 in psoriasis.

An effect of 1α,25(OH)$_2$ vitamin D_3 was seen in 88% of all coverslips and usually occurred at physiological concentrations. Higher concentrations were required if cells had been exposed to another agonist, which emphasizes the need to examine one agonist at a time.

The intracellular free calcium concentration was not quantifiable in the majority of coverslips for the following reasons: the baseline was sloping or erratic; the fluorescent response to 1α,25(OH)$_2$ vitamin D_3 was greater than F_{max}; the baseline fluorescence was less than F_{min}. A sloping baseline may be explained by gradual leakage of the dye from the cells, by photobleaching or by cell shedding. An erratic baseline may be caused by small movements of the coverslip within the cuvette. The problem of the fluorescence being either greater than F_{max} or less than F_{min} is probably explained by sequestration of the dye within the cells.[16] This technique will reliably detect changes in intracellular free calcium but is less suitable for quantitative data.

As yet there is little published work on intracellular free calcium in human keratinocytes. One report suggested that an increase in free intracellular calcium occurred with differentiation.[17] Another study[18] demonstrated that murine keratinocytes respond to 1OH vitamin D_3, 24,25(OH)$_2$ vitamin D_3 and 1α,25(OH)$_2$ vitamin D_3 with an acute rise in intracellular free calcium lasting for several minutes, the three compounds being equipotent. However, the calcium estimations were performed on keratinocytes in suspension which responded only to relatively high concentrations of 1α,25(OH)$_2$ vitamin D_3 (5×10^{-8} M). In our study, adherent sheets of keratinocytes responded to physiological concentrations of 1α,25(OH)$_2$ vitamin D_3 (10^{-11}–10^{-9} M), often with rapid transient increases in intracellular free calcium. These differences may be explained by the fact that keratinocytes in culture show complex cell-to-cell communication which would be absent in cell suspensions, and in addition cells in suspension suffer surface receptor damage during disaggregation and dispersion.

The response to 1α,25(OH)$_2$ vitamin D_3 was sometimes delayed for several minutes, but not lost, with the addition of 10^{-4} M cycloheximide, a protein synthesis inhibitor effective in keratinocytes.[19] The 'spiking' in calcium seen with 1α,25(OH)$_2$ vitamin D_3 was also seen in response to foetal bovine serum. Spiking in intracellular free calcium has recently been reported in other cells and in synchronized cell cultures.[20] Desmosomal junctions are known to be a normal feature of cultured keratinocytes, so cell-to-cell communication may have contributed to the oscillations which were often extremely large. Spiking in intracellular calcium could be a means of preventing cellular calcium toxicity which might occur in response to a prolonged stimulus. A spiking mechanism would allow intracellular calcium to be normal for a large percentage of the time during cell stimulation.[20]

The timing of the response to 1α,25(OH)$_2$ vitamin D_3 and its independence of protein synthesis imply that the hormone does not act via stimulation of a nuclear steroid receptor resulting in protein synthesis. The finding of a relatively acute effect of 1α,25(OH)$_2$ vitamin D_3 on intracellular free calcium has been made for other cells.[12,13]

The source of the increase in intracellular free calcium now needs to be explored. In B16 melanoma cells we were able to establish that 1α,25(OH)$_2$ vitamin D_3 increased intracellular free calcium by causing an influx of extracellular free calcium with no apparent effect on intracellular stores.[12] In addition our preliminary data that keratinocytes could also respond to unhydroxylated vitamin D_3, albeit at much higher concentrations, requires further investigation to determine which vitamin D_3 metabolites can produce responses. There was a marked tachyphylaxis in the response to 1α,25(OH)$_2$ vitamin D_3 which could be explained by a temporary exhaustion of intracellular stores of calcium.

The relationship of vitamin D metabolism to the pathogenesis of psoriasis is controversial. Hypocalcaemia has been reported to precipitate generalized pustular psoriasis of von Zumbusch.[21] One study has demonstrated abnormal vitamin D metabolism in patients with psoriasis *in vivo*,[22] but this has not been confirmed by others.[3] *In vitro*, cultured fibroblasts derived from psoriatic patients are partially resistant to the antiproliferative effects of 1α,25(OH)$_2$ vitamin D_3,[23] but whether the sensitivity to $1\alpha,25(OH)_2$ vitamin D_3 is altered in psoriatic keratinocytes is disputed.[24,25]

In summary, the finding that $1\alpha,25(OH)_2$ vitamin D_3 can increase intracellular free calcium in normal cultured keratinocytes begs the question of the relevance of this finding to normal and abnormal keratinocyte biology. With the recent evidence of vitamin D_3 metabolites improving psoriasis we would propose that exposure of epidermal keratinocytes to vitamin D_3 metabolites may increase keratinocyte calcium and induce differentiation. We offer this as a hypothesis for investigation which may contribute to our knowledge of vitamin D_3 biology and to our understanding of psoriasis.

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Tissue kallikrein and kininogen in human sweat glands and psoriatic skin

M.T.POBLETE, N.J.REYNOLDS,* C.D.FIGUEROA, J.L.BURTON,* W.MULLER-ESTERL† AND K.D.BHOOLA

*Departments of Pharmacology and *Dermatology, University of Bristol, U.K.*
†Department of Pathobiochemistry, Institute of Physiological Chemistry, University of Mainz, Germany

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Summary

The cellular localization of immunoreactive tissue kallikrein and kininogen was studied in normal and psoriatic human skin. Immunoreactivity to both enzyme and substrate was observed in secretory granules of the dark cells in the secretory fundus (acinus) of the sweat glands. Double immunostaining revealed a segmental distribution of the two antigens. Each acinar section contained either tissue kallikrein or kininogen. However, there appeared to be a junctional zone in which both were present, but in separate dark cells. Immunoreactivity for both antigens was also observed in close apposition to the luminal microvilli of the duct cells. No specific immunostaining was seen in sebaceous glands, hair follicles, keratinocytes and other cells of the secretory unit such as myoepithelial or clear cells. In psoriatic skin there were in addition many neutrophils immunoreactive for tissue kallikrein in the epidermis and psoriatic scales. Mitogenic action of kinins may account to some extent for the characteristic accelerated turnover of epidermal cells in psoriasis and locally applied kinin antagonists may prove of value in the treatment of this disease.

The presence of a kinin-forming enzyme in normal human eccrine sweat was first reported in 1958.[1] Later, this kininogenase enzyme in sweat[2] was shown to closely resemble a family of proteases known as kallikreins.[3] However, these findings were questioned by Frewin *et al.*,[4] because of their inability to detect kinins or kininogenase activity in normal human sweat. Further differences emerged when a kininogenase, sensitive to inhibition by soya bean trypsin inhibitor but immunologically distinct from plasma or tissue kallikrein, was purified from extracts of human skin.[5] Subsequently, using a specific radioimmunoassay, Mann *et al.* [6] reported the presence of a tissue kallikrein in human sweat, and this was later partially purified by Hibino *et al.*[7] More recently, variations in the amount of immunoreactive tissue kallikrein have been reported in sweat collected from different regions of the body, the highest levels being found in samples obtained from the trunk and forehead.[8]

The role of kinins in inflammatory skin disease is poorly understood, but extracts prepared from the epidermal scales of psoriatic patients have been shown to contain a tissue kallikrein-like enzyme which forms a kinin from bovine L-kininogen.[9] We have now examined the cellular localization of tissue kallikrein and its substrate, kininogen, in human skin biopsies obtained from both normal volunteers and patients with psoriasis.

Methods

Tissue samples

Skin biopsies (4-mm punch) were taken under local anaesthetic (2% lignocaine with 1:80,000 adrenaline) from the upper arm of six normal volunteers and from the lesional skin of six patients with psoriasis. They were fixed in formal-saline (10% v/v) at room temperature for 24–48 h. The tissue blocks were dehydrated in a graded series of ethanol and embedded in paraffin wax. Sections 4–5 μm thick were mounted on glass slides coated with polylysine (Sigma Chemicals, Poole, Dorset, U.K.).

Antisera

Tissue kallikrein. Immunocytochemistry was performed with three polyclonal antibodies raised against human urinary kallikrein: two rabbit antisera[10,11] and a goat antiserum (Protogen AG, Switzerland).

Kininogen. A polyclonal antibody raised against the whole L-kininogen molecule[12] was used in this study.

Correspondence: Dr K.D.Bhoola, Department of Pharmacology, University of Bristol, Bristol BS8 1TD, U.K.

Immunostaining

Two different immunostaining procedures were carried out: the peroxidase anti-peroxidase method (PAP) as described previously[13] and the immunogold silver staining (IGSS) method.

PAP method. Skin tissue sections were dewaxed with xylene and rehydrated through graded series of ethanol. The sections were treated with absolute methanol and 3% (v/v) hydrogen peroxide to block endogenous pseudoperoxidase activity[14] and then rinsed in Tris-HCl (0·05 mol/l)-sodium chloride (0·15 mol/l) buffer (pH 7·8). The samples were then incubated with anti-tissue kallikrein (1:300–1:5000) or anti-kininogen (1:400–1:1000) antibody for 18 h. When a goat primary antibody was used, the second step included incubation with rabbit anti-goat IgG serum (1:1000) (Sigma) for 30 min. Swine anti-rabbit IgG and PAP complexes (Dakopatts, Denmark, Copenhagen) were used at dilutions of 1:40 and 1:100, respectively. All incubations were performed at 22°C in a water bath that was used as a humidifier. Peroxidase activity was demonstrated with 0·1% 3,3 diaminobenzidine tetrahydrochloride in the presence of 0·05% hydrogen peroxide. Finally, the sections were counterstained with haematoxylin.

IGSS method. The tissue sections were dewaxed, rehydrated as previously described and rinsed twice for 5 min each in PBS (0·01 mol/l pH 7·2). They were then immunostained by incubation with: (i) 5% non-immune goat serum for 20 min; (ii) anti-tissue kallikrein serum (1:5000) (Protogen A.G., Switzerland) at 22°C for 18 h; (iii) rabbit anti-goat IgG (1:1000) (Sigma) for 30 min; (iv) gold-labelled goat anti-rabbit IgG (Janssen Biotech, Belgium) (1:40) for 1 h; (v) silver enhancement for 10–20 min. The silver enhancement reagent (Janssen) was prepared by mixing equal amounts of enhancer and initiator solutions. Finally, the slides were washed thoroughly with distilled water, counterstained with 1% neutral red and then mounted in DPX.

Double immunostaining. A double immunostaining method[15] was used to demonstrate tissue kallikrein and kininogen in the same sections, using two different chromogens. Tissue sections were immunostained for tissue kallikrein as described previously to develop the first colour (brown). Before the second immunostaining procedure, an elution step was performed. Sections were washed for 1 h with several changes of glycine-HCl (0·2 mol/l)-sodium chloride (0·5 mol/l, pH 2·3) and 10% dimethylformamide (Sigma) in Tris-HCl buffer (0·05 mol/l, pH 7·8).[15] The sections were then incubated with rabbit anti-kininogen serum (1:200), anti-rabbit IgG (1:40 v/v) and PAP (1:100 v/v). Peroxidase activity was developed with 4-chloro-1-naphthol (Sigma). Finally, the sections were covered with a glass coverslip using a solution of glycerol and Tris-HCl-saline (1:1 v/v).

Controls. The specificity of the immunostaining was checked by prior absorption of the tissue kallikrein antisera with purified human urinary kallikrein (50 μg/ml). The antiserum against L-kininogen was absorbed with purified human L-kininogen (20–50 μg/ml). Other controls included omission of the specific antiserum and replacement by non-immune serum.

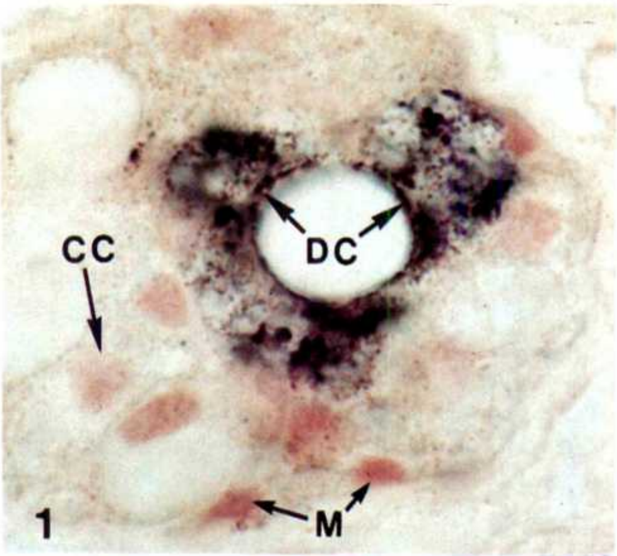
Results

Normal skin

Tissue kallikrein. A granular pattern of immunostaining was observed in the acinar dark cells arranged around the lumen of the secretory unit of sweat glands (Fig. 1). Other components of the secretory unit such as the clear cells and myoepithelial cells showed no immunostaining. Staining was also seen as an intense line along the luminal microvilli of the duct cells up to its opening on the surface of the skin (Fig. 2). There was no evidence of specific staining in the sebaceous glands, hair follicles, keratinocytes, dermal vessels or in the interstitial tissue space. Both PAP and IGSS methods gave identical results.

Kininogen. An intense immunoreactivity was observed in the dark cells of the acinus which was different from those containing tissue kallikrein (Fig. 3). As with tissue kallikrein, no specific immunoreactivity to kininogen was seen in other cells of the acinus or in the interstitial tissue space but staining was observed in the capillaries. Deep staining for kininogen was observed in apposition to the microvilli of the luminal duct cells.

Double immunostaining. The use of the double-immunostaining method allowed the visualisation of both antigens in the same section. Serial sections showed that there was a segmental distribution of the two antigens. Individual sections of the secretory fundus contained either one or other of the antigens, but there appeared to be a junctional region of the fundus in which both antigens were present in separate dark cells (Fig. 4).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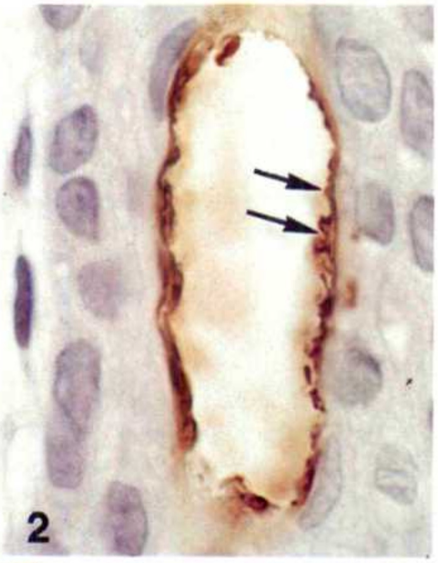
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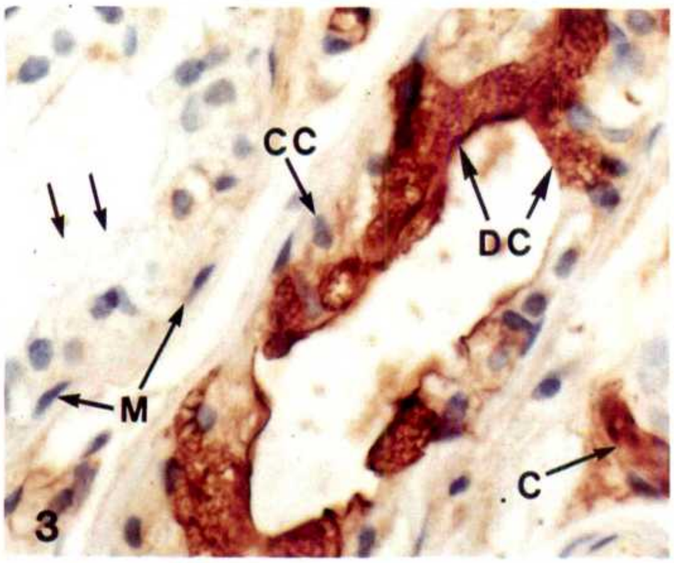
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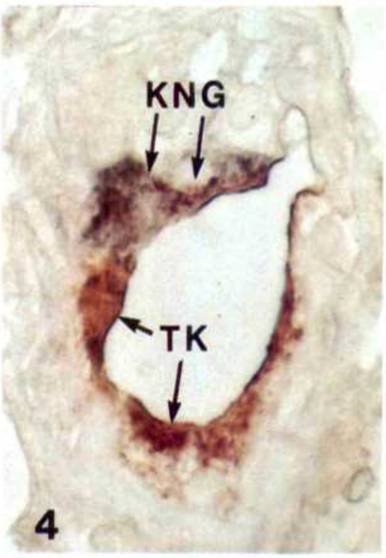
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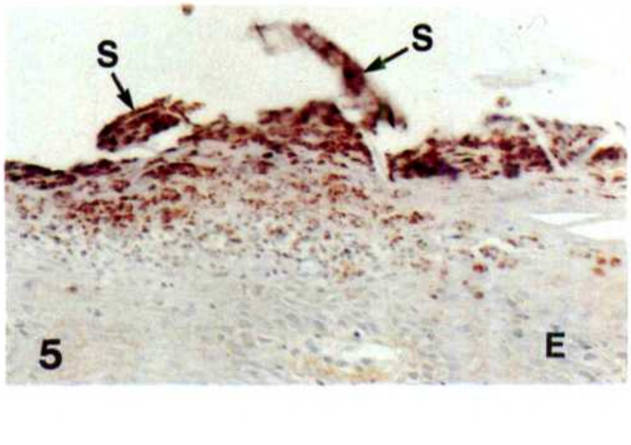
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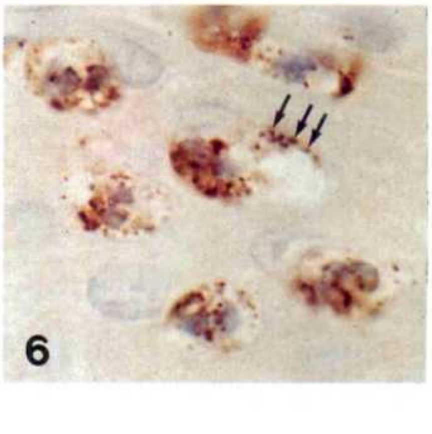
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Psoriatic skin

Tissue kallikrein. In addition to the immunoreactivity present in the dark cells of the secretory fundus, a clear granular pattern of immunostaining was observed in the cytoplasm of neutrophils that had migrated into the epidermis and had accumulated in the psoriatic scales (Fig. 5 and 6). In the epidermis the neutrophils orientated either individually or in clusters (Fig. 6).

Kininogen. A distribution similar to that described for the normal skin was observed in the biopsies from psoriatic patients.

Discussion

Following the description of a bradykinin-forming enzyme in human sweat by Fox and Hilton,[1] several investigators proposed a role for kinins in sweat gland function. However, the precise segment of the gland and the cells that contain the enzyme(s) and the substrate (kininogen) necessary for the formation of kinins were not clearly delineated. Using immunocytochemical techniques we have now localized tissue kallikrein and its substrate, kininogen, in the dark cells of the secretory fundus of sweat glands. Furthermore, using double-immunostaining procedures it was found that tissue kallikrein and kininogen were located in distinct regions of the fundus. In the junctional zone the enzyme and substrate were each seen in separate dark cells, which were intermingled (Fig. 7). A similar anatomical relationship has been found in the human kidney.[13]

Our observation that the secretory granules containing tissue kallikrein or kininogen are orientated to the apical end of the cell suggests that the primary secretion of the enzyme and substrate into the acinar lumen probably occurs by exocytosis. The structural organisa-

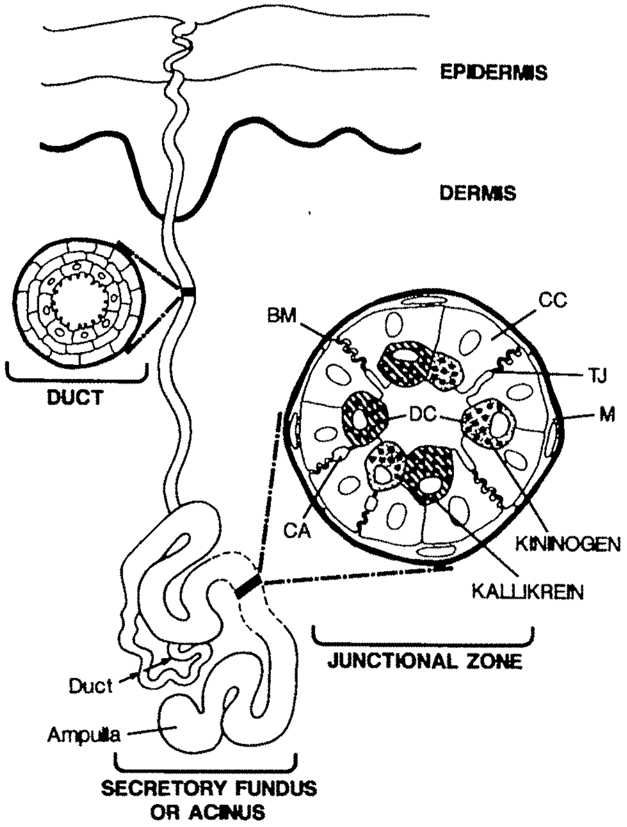

Figure 7. Diagrammatic representation of the structural and cellular appearance of the human sweat gland. BM, basal membrane; M, myoepithelial cell; CC, clear cell; DC, dark cell; TJ, tight junction; CA, cannaliculus.

Figure 1. Tissue kallikrein, normal skin. Intense immunostaining in the dark cells (DC) of the secretory fundus. Clear cells (CC) and myoepithelial cells (M) remain unstained. IGSS method × 2300.

Figure 2. Tissue kallikrein, normal skin. Arrows indicate immunoreactivity visualised along the luminal microvilli of the duct cells. PAP method × 2000.

Figure 3. Kininogen, normal skin. Immunoreactive kininogen is contained in dark cells (DC) of the secretory fundus. Arrows point to unstained DC which showed immunoreactivity for tissue kallikrein in the next section. Immunoreactive kininogen is also observed in the lumen of a capillary (C). CC, clear cells; M, myoepithelial cell. PAP method × 1000.

Figure 4. Tissue kallikrein–kininogen double immunostaining, normal skin. Junctional region of the secretory fundus in which the dark cells containing tissue kallikrein (TK, brown) and kininogen (KNG, purple) intermingle. PAP method × 1200.

Figure 5. Tissue kallikrein, psoriatic skin. Dense neutrophil infiltrates (arrows) invade the epidermis (E) and psoriatic scales (S). Most of the neutrophils contain immunoreactive tissue kallikrein. PAP method × 500.

Figure 6. Tissue kallikrein, psoriatic skin Immunoreactive tissue kallikrein is localized in the cytoplasmic granules of neutrophils (arrows). PAP method × 2500.

tion of the dark cells and intercellular tight junctions[16] is such that during active sweat formation backward transport of macromolecules[17] into the interstitial tissue space is unlikely[16]. In our study we found no immunoreactive tissue kallikrein or kininogen beyond the outer surface of the basal lamina of the secretory epithelium. During prolonged stimulation dark cells that cannot sustain secretion are discarded from the secretory epithelium.[18] Disintegration of dark cells causes large intercellular gaps to appear, thereby exposing the myoepithelial cells of the secretory coil to the contractile action of kinins, in addition to the neuronally released acetylcholine. In the recovery phase following intense sweating, the dead cells are replaced by direct division of remaining epithelial cells. The known mitogenic property of kinins[19–21] may induce mitotic activity in the secretory epithelium after recovery from sweating.

The iontophoretic application of bradykinin produces an abrupt fall of sweat osmolality.[22] Moreover, kallidin (lys-bradykinin) increases sodium absorption across cultured sweat epithelium in a manner that seems to be independent of the generation of prostaglandins.[23] However, these effects were only observed when the peptide was added to the basolateral and not to the apical face of the monolayers.[23] During thermal stimulation of sweat glands, small clear vesicles appear on the luminal surface that constitute a resorptive mechanism for the movement of molecules to the serosal side.[24] In addition, the intercellular spaces between the luminal cells of the duct widen during sweating. If, therefore, kinins are formed in the duct lumen, permeation to the serosal side of the luminal cells could modulate the excretion of sodium and water by the gland, assuming they survive the action of kininases. A kininase with properties similar to those of angiotensin I converting enzyme (kininase) has been recently partially purified from human sweat by Hibino *et al.*[7] Sideness-preference (basolateral versus apical) for bradykinin and kallidin in ion transport has also been described using isolated cortical collecting ducts from rabbit kidneys. At this level kinins inhibit sodium absorption and arginine vasopressin-mediated water resorption.[25,26] Furthermore, the formed kinins could enhance the contractile movements of the microvilli in order to increase propulsion of sweat to the epidermis.

One of the characteristic histopathological features of psoriasis is the presence of a dense infiltrate of polymorphonuclear leucocytes, that may result in microabscess formation within the stratum corneum.[27] Psoriatic scales contain leucotactic substances that probably correspond to complement-associated peptides generated by the action of proteolytic enzymes.[28,29] We have shown that circulating neutrophils which infiltrate the epidermis of these patients contain immunoreactive tissue kallikrein.[30] In addition, neutrophils possess receptors for H-kininogen and contain immunoreactive L- and H-kininogens.[31–32] Kininogen is increased in several inflammatory diseases including rheumatoid arthritis[33] and psoriasis.[34] As kininogens are synthesized by hepatocytes in the liver, cytokines or similar mediators produced locally in inflamed tissue may be responsible for switching on the hepatic synthesis and secretion of the kininogen into the circulation.

Kinins are believed to act as mitogens, stimulating DNA synthesis and cell division.[19–21] Intradermal occlusions of the sweat gland duct unit[35,36] together with the migration of neutrophils into pustules and microabscesses may result in an increased formation of the peptide. The higher kinin levels could in part account for the increased mitotic activity seen in the psoriatic epidermis. It is therefore possible that kinins contribute to the pathogenesis of psoriasis, and locally applied kinin antagonists may prove of therapeutic value.

Acknowledgments

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An immunohistological study of desmosomes in Darier's disease and Hailey–Hailey disease

SUSAN M.BURGE AND D.R.GARROD*

Department of Dermatology, Slade Hospital, Oxford

**Cancer Research Campaign Medical Oncology Unit, University of Southampton, Southampton General Hospital*

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Summary

The immunocytochemical distribution of desmosomal components was determined in involved skin from eight patients with Darier's disease, five patients with Hailey–Hailey disease and two patients with transient acantholytic dermatosis as well as skin from four normal controls. Sections were stained using monoclonal antibodies to the desmosomal proteins dp1 and dp2 (desmoplakins) and the desmosomal glycoproteins dg1 (desmoglein), and dg2 and dg3 (desmocollins). There was normal expression of desmosomal proteins and glycoproteins at the periphery of the keratinocytes in the perilesional skin in Darier's disease, in Hailey–Hailey disease and in transient acantholytic dermatosis. In the lesional skin there was reduced expression of desmosomal proteins and glycoproteins in the basaloid 'buds' at the base of the lesions, but there was bright diffuse staining of the acantholytic cells. Focal intracellular staining was detected within many of the acantholytic keratinocytes in Hailey–Hailey disease and within some of these cells in Darier's disease. Suction blisters were used to induce fresh acantholysis in lesional skin in Darier's disease and clinically uninvolved skin in Hailey–Hailey disease. The results indicated that acantholysis precedes the development of intracellular staining.

Although there are immunopathological abnormalities in the distribution of desmosomal proteins and glycoproteins in both Darier's disease and Hailey–Hailey disease, the changes are probably secondary to internalization of desmosomal components with breakdown and redistribution of antigens rather than a primary deficiency in the synthesis of these proteins. Focal internalization was more widespread in Hailey–Hailey disease than in Darier's disease and the differences in the distribution of desmosomal components in these diseases confirm that they are distinct entities.

Darier's disease (keratosis follicularis) and Hailey–Hailey disease (benign familial chronic pemphigus) are dominantly inherited genodermatoses which share some clinical and many histological features. Family studies and pathological observations indicate that the diseases are separate entities.[1] In both diseases there is a loss of cohesion between epidermal cells and typical histological features include focal areas of acantholysis as well as a characteristic dyskeratosis.[2,3]

Keratinocyte adhesion is mediated by specialized junctions, the desmosomes along with other adhesion molecules on the cell surface. The desmosomes interact with the keratin intermediate filaments of the cytoskeleton. Thus, desmosomes both join keratinocytes together and provide links between their cytoskeletons. Ultrastructural studies in both diseases have demonstrated detachment of keratin filaments from desmosomes, perinuclear aggregations of keratin filaments, membrane changes and loss of desmosomes.[2–9] Despite these detailed studies the primary abnormalities are unknown and the sequence of events is uncertain.

Acantholysis in one or both diseases may be secondary to abnormalities in the structure or organization of the desmosomes. Desmosomes are 0·1–0·5 μm in diameter with a well-defined structure. Dense plaques 10–15 nm thick are present on the cytoplasmic face of the keratinocyte membranes and keratin intermediate filaments approach the inner face of the plaque, but are separated from it by a zone containing fine filamentous material, which may anchor the keratin filaments to the plaque.[10–13]

In stratified epithelia two groups of desmosomal proteins have been identified: non-glycosylated and glycosylated. A number of the desmosomal proteins and glycoproteins have been characterized and using specific antisera the different components have been localized by immunoelectron microscopy. The desmosomal proteins (dps) include a pair of high molecular weight proteins,

Correspondence: Dr S.M.Burge, Department of Dermatology, Slade Hospital, Oxford OX3 7JH, U K.

dp1 (desmoplakin 1; M_r 250,000) and dp2 (desmoplakin 2; M_r 215,000), which are probably localized within the clear zone between the plaque and the insertion of the intermediate filaments.[11,13] Desmocalmin (M_r 240,000), a calmodulin and keratin filament-binding protein, is another high molecular mass constituent of the plaque region.[14] In addition there are a pair of lower molecular weight proteins, dp3 (band 5 protein, plakoglobin; M_r 83,000) and dp4, a basic polypeptide (band 6 protein; M_r 75,000). Dp3, which is found in adherens junctions as well as desmosomes, is localized in the plaque close to the plasma membrane[11,15,16] and extends into the plaque. Dp4 is probably present in the cytoplasm adjacent to the plaque.[11,13,17] Recently a high molecular mass protein (M_r 680,000), desmoyokin, has been described associated with the circumference of the desmosomal plaque.[18]

The desmosomal glycoprotein (dgs) which are probably important in the formation and maintenance of stable cell adhesion, include a group of related glycoproteins, dg1, (desmoglein 1; M_r 175,000–164,000) and a pair of lower molecular weight glycoproteins dg2 (desmocollin 1; M_r 130,000) and dg3 (desmocollin II; M_r 115,000). Both groups are transmembrane glycoproteins which contribute to the structure of intercellular material and the cytoplasmic plaque.[11-13,19-22]

An investigation into the distribution of desmosomal components in Darier's disease and Hailey–Hailey disease might provide insight into the pathogenesis of these genodermatoses and contribute to our understanding of the differences between them. The aims of our study were to determine, using monoclonal antibodies, the immunocytochemical distribution of desmosomal components in clinically normal and lesional skin in Darier's disease and to compare and contrast the findings with those in Hailey–Hailey disease. In addition we studied transient acantholytic dermatosis (TAD), an acquired dermatosis which has many histological similarities to Darier's disease.

Methods

Local ethical committee approval was obtained for this study and informed consent was given by the patients.

Tissues

Frozen material. Punch biopsies of typical lesions were obtained from eight patients with histologically confirmed Darier's disease (five males and three females); and five patients with histologically confirmed Hailey–Hailey disease (four males and one female). One patient with Darier's disease was taking etretinate 50 mg per day. Frozen specimens were obtained from one male patient with TAD. The specimens were snap frozen and stored in liquid nitrogen until frozen sections were cut. Four specimens of normal skin collected during routine surgical procedures were snap frozen and stored.

Paraffin-embedded material. Specimens were also obtained from the Department of Pathology, John Radcliffe Hospital, Oxford. These specimens had been fixed in 10% formalin and processed into paraffin using routine techniques. These comprised specimens from Darier's disease (three), Hailey–Hailey disease (two) and TAD (two).

Suction blisters

In the skin of normal individuals suction induces subepidermal blister formation without intraepidermal cleavage, but in Hailey–Hailey disease suction causes intraepidermal cleavage in histologically normal skin.[23] In Darier's disease, suction causes subepidermal splitting in perilesional skin, but increases intraepidermal splitting and acantholysis in lesional skin.[24] This technique was used to produce fresh cleavage and acantholysis in two patients with Darier's disease, one patient with Hailey–Hailey disease and one normal individual. Blisters were raised as previously described[24] using a pressure of 200 mmHg below atmospheric pressure. Biopsies of the blisters were snap frozen.

Antibodies

Monoclonal antibodies to the desmosomal components of dp1/2 (11-5F), dg1 (33-3D) and dg2/3 (52-3D) were used on frozen material and a second monoclonal antibody to dg1 (32-2B) was used on formalin-fixed material. The characteristics of these antibodies are shown in Table 1. Optimum working dilutions were neat for 32-2B; 1:2 in phosphate-buffered saline (PBS), pH 7·2, for 33-3D and 52-3D; 1:20 in PBS for 11-5F.

Immunostaining of frozen material

Frozen sections (8 μm thick) were cut, placed on multiwell slides and air-dried for 30 min. The sections were incubated for 1 h with antibody, washed in PBS for 3 × 10 min and incubated for a further hour with rabbit

Table 1. Monoclonal antibodies to desmosomal proteins and glycoproteins

Designation	Ig subclass (mouse)	Specificity*	Reference
11-5F‡	IgG1	dp1 + 2 (desmoplakins)	25
32-2B†‡	IgG2a	dg1 (desmoglein)	20
33-3D†	IgM	dg1 (desmoglein)	21, 26 and unpublished observations
52-3D†	IgG1	dg2 + 3 (desmocollins)	22

* Determined by immunoblotting on bovine nasal epidermis.

† All three antiglycoprotein antibodies have been shown to recognize the membrane-protected (cytoplasmic) domains of the molecules.[21,22,26]

‡ Localized to desmosomes by immunoelectron microscopy (unpublished observations).

anti-mouse immunoglobulin fluorescein-labelled conjugate [Dako] diluted 1/50 in PBS. All incubations were carried out at room temperature. Slides were washed in PBS for 3 × 10 min, mounted in buffered glycerol and examined by fluorescence microscopy. Control sections were incubated with PBS instead of the primary antibody and non-specific autofluorescence was assessed by examining sections which had been incubated solely in PBS.

Immunostaining of paraffin sections

This was carried out as previously described[21] and sections were lightly counterstained with haematoxylin.

Detergent extraction

Selected sections were pre-incubated with 0·5% Triton X 100 in PBS for 45 min to remove any soluble, unbound protein and the sections were washed in PBS before staining with anti-desmosomal antibodies.

Results

Normal skin

Anti-dp1/2 (11-5F) (frozen sections). There was no staining of the bases of the basal cells where they were in contact with the basement membrane zone (BMZ). Elsewhere in the epidermis there was fine stippled staining of the periphery of epidermal cells and complete absence of cytoplasmic staining (Fig. 1a). The intensity of the stain was weakest between the lateral surfaces of the basal cells but there was strong staining of the upper poles of the basal cells where they were in contact with the suprabasal cells. Uniformly bright staining around the periphery of the cells throughout the rest of the epidermis extended to the granular layer. The discrete particulate nature of the staining was always maintained. Staining was not detected in the stratum corneum.

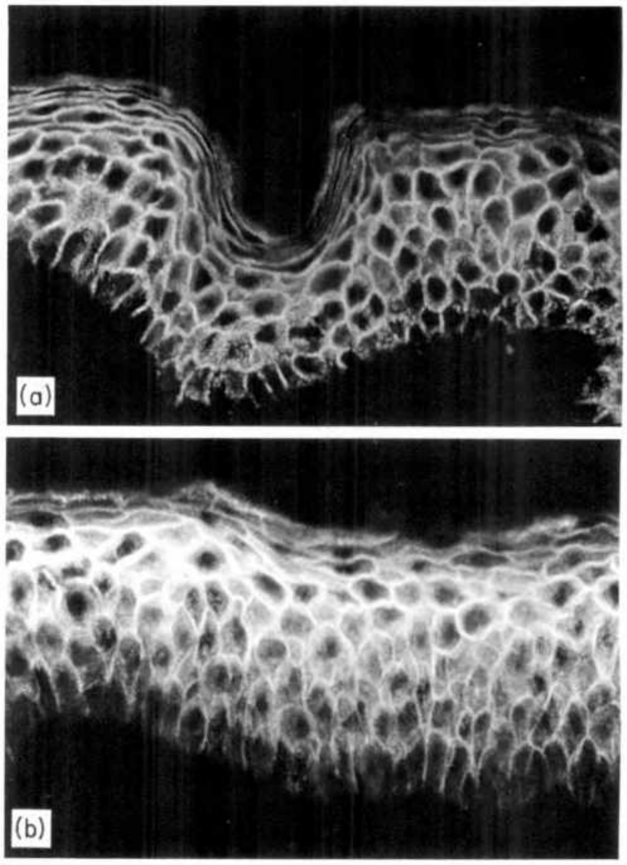

Figure 1. Normal skin. (a) Immunostaining of a frozen section with anti-dp1/2. The base of the basal cells in the region of the hemidesmosomes is not stained but there is fine stippled staining around the keratinocytes in the rest of the epidermis (× 700). (b) Immunostaining of a frozen section with anti-dg2/3. There is punctate staining at the periphery of the keratinocytes but, as with anti-dp1/2, there is no reactivity with the cell membrane in the region of the hemidesmosomes. Staining is weak in the basal layer but thereafter gradually increases in intensity and is maximal around the keratinocytes in the high suprabasal and granular layers (× 700).

Anti-dg1 (33-3D: frozen sections; 32-2B: paraffin sections), and anti-dg2/3 (52-3D: frozen sections). These antibodies also stained the periphery of the epidermal cells in a punctate linear pattern sparing the bases of the basal cells (Fig. 1b). The staining with the dg antibodies was weaker in the basal layer than it was with the dp antibody but the intensity of staining increased higher in the epidermis and was maximal around the higher suprabasal cells and in the granular layer. There was no staining in the stratum corneum and no cytoplasmic staining. For illustrations of staining with 32-2B see Vilela *et al.*[20]

Darier's disease

Anti-dp 1/2 (11-5F) (frozen sections). There was a normal peripheral pattern of staining around the keratinocytes in perilesional skin. In the lesional skin there was weak staining around the basaloid cells in the 'buds' at the base of the lesion. In some of the acantholytic cells the stain was concentrated within the cytoplasm in a thick, homogeneous peripheral 'doughnut' rim sparing the nucleus while in others the entire cell was stained (Fig. 2a). The stain was often brighter in these cells than in the surrounding unaffected skin. Sometimes it was possible to discern a granular element to this staining when the focusing was adjusted. In a few cells there was a second perinuclear rim of stain clearly separated from and inside the peripheral rim. There was bright stain outlining or within the corps ronds in the granular layer, but no staining in the keratotic plug.

Anti-dg1 (33-3D: frozen sections); 32-2B: paraffin sections), anti-dg2/3 (52-3D: frozen sections). There was a normal peripheral pattern of staining around the epidermal cells in the perilesional skin. In the lesional skin the results were essentially similar to those obtained with anti-dp1/2 with bright diffuse staining or thick periph-

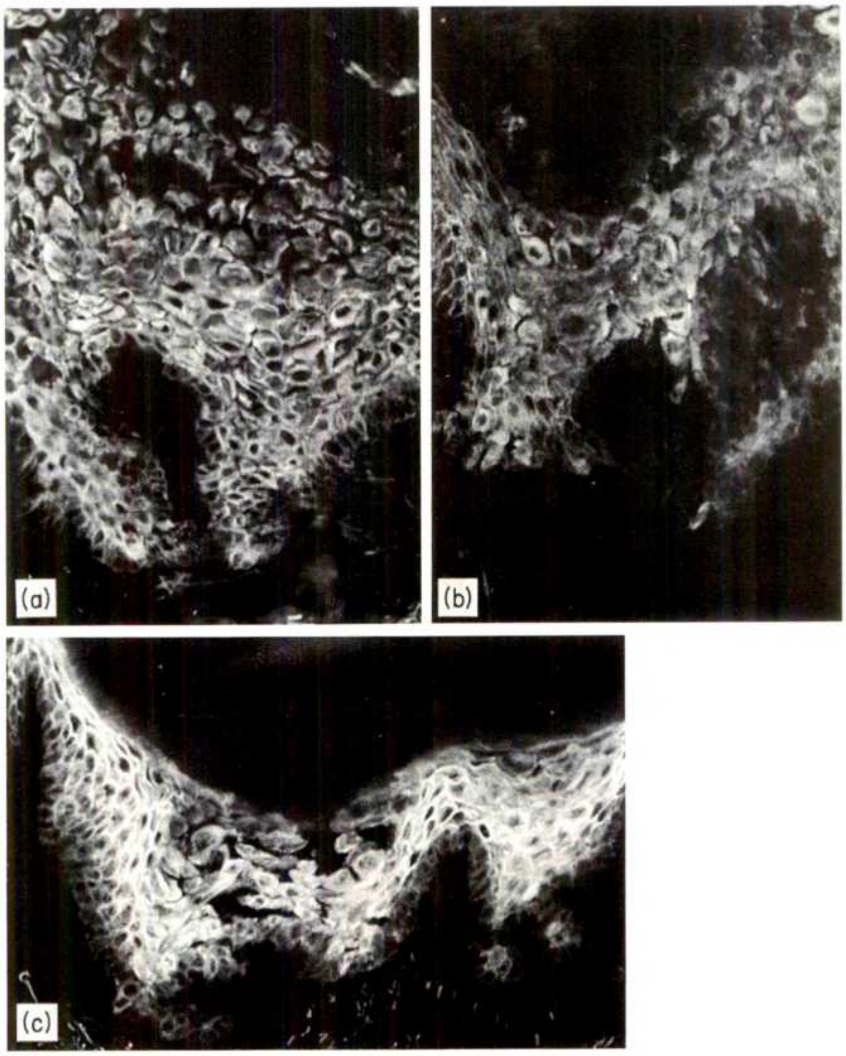

Figure 2. Darier's disease. (a) Immunostaining of a frozen section with anti-dp1/2. In some of the acantholytic cells the stain is concentrated within the cytoplasm in a thick, homogeneous peripheral 'doughnut' rim sparing the nucleus while in others the entire cell is stained. The stain is often brighter in these cells than in the surrounding unaffected skin (× 700). (b) Immunostaining of a frozen section with anti-dg1. In the lesional skin there is weak staining around the basaloid cells in the 'buds' at the base of the lesion but an intense ring of stain or diffuse staining in the majority of the acantholytic cells (× 700). (c) Immunostaining of a frozen section with anti-dg2/3. The desmosomal glycoproteins are expressed normally in perilesional uninvolved skin. In some of the acantholytic cells the stain is concentrated within the cytoplasm in a thick, homogeneous peripheral 'doughnut' rim sparing the nucleus, but in others a double ring of cytoplasmic staining is apparent or the entire cell was stained.(× 700)

eral staining of the majority of the acantholytic cells (Figs 2b and c). Occasionally it was possible to detect a focal area of more intense staining adjacent to the cell nucleus. The reduction in the intensity of the staining throughout the basal 'buds' beneath the keratotic papule was more marked than with anti-dp1/2 and sometimes there was no detectable peripheral staining around the cells in these buds.

Hailey-Hailey disease

Anti-dp1/2 (11-5F) (frozen sections). There was normal discrete, fine punctate staining of the peripheries of epidermal cells in the perilesional skin. In the lesional skin there were abnormal bright clumps of peripheral stain around many of the basal cells and bright diffuse staining of most of the acantholytic cells (Fig. 3a). It was possible to discern a particulate element to this diffuse staining when the focusing was adjusted. There was a normal peripheral pattern of stain around the relatively cohesive cells in the blister roof.

Anti-dg1 (33-3D: frozen sections), (32-2B: paraffin sections), anti-dg2/3 (52-3D: frozen sections). In the perilesional skin there was a normal pattern of peripheral staining around the epidermal cells. In Hailey–Hailey disease, as in Darier's disease, the cells of the basal buds were weakly stained or negative. There was diffuse stain over the majority of the acantholytic cells (Fig. 3b). In the paraffin section incubated with 32-2B dense intracellular areas of perinuclear staining were apparent within the acantholytic cells (Fig. 3c). There was also

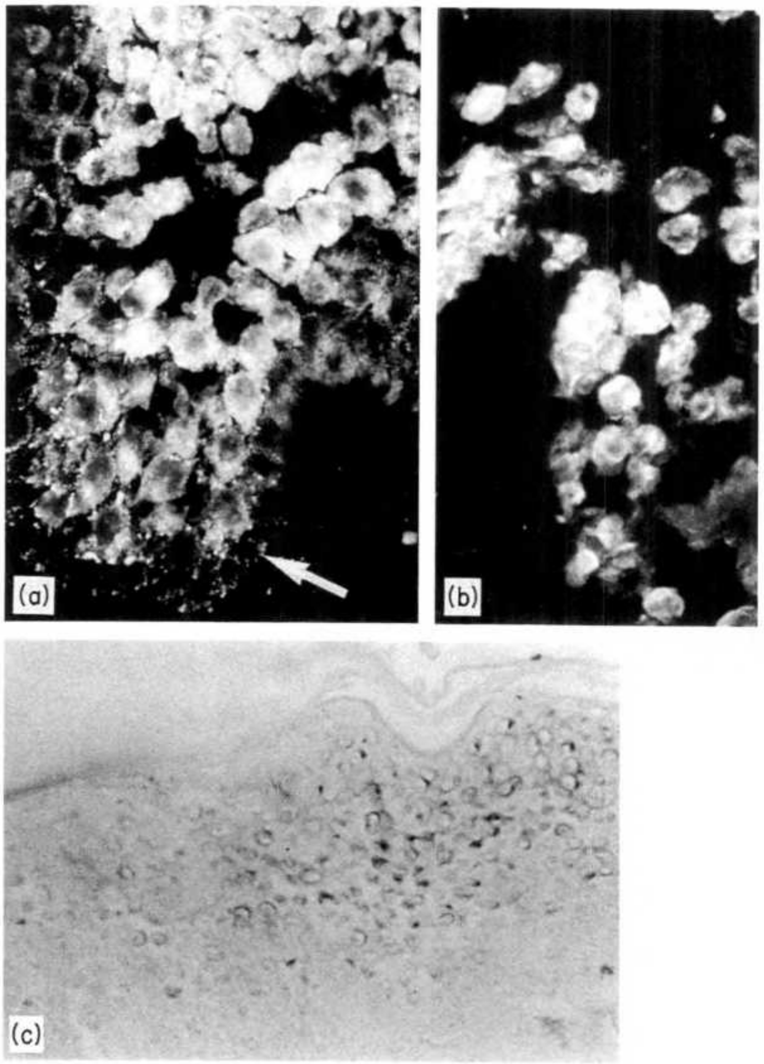

Figure 3. Acantholytic cells in Hailey–Hailey disease. (a) Immunostaining of a frozen section with anti-dp1/2. In the lesional skin there are abnormal bright clumps of peripheral staining around many of the basal cells [→] and bright diffuse staining of most of the acantholytic cells (× 700). (b) Immunostaining of a frozen section with anti-dg1. There is intense diffuse staining over the majority of the acantholytic cells (× 700). (c) Immunostaining of paraffin section with anti-dg1. Cytoplasmic staining was apparent within many of the acantholytic cells as well as within the keratinocytes in the roof of the lesion (× 400).

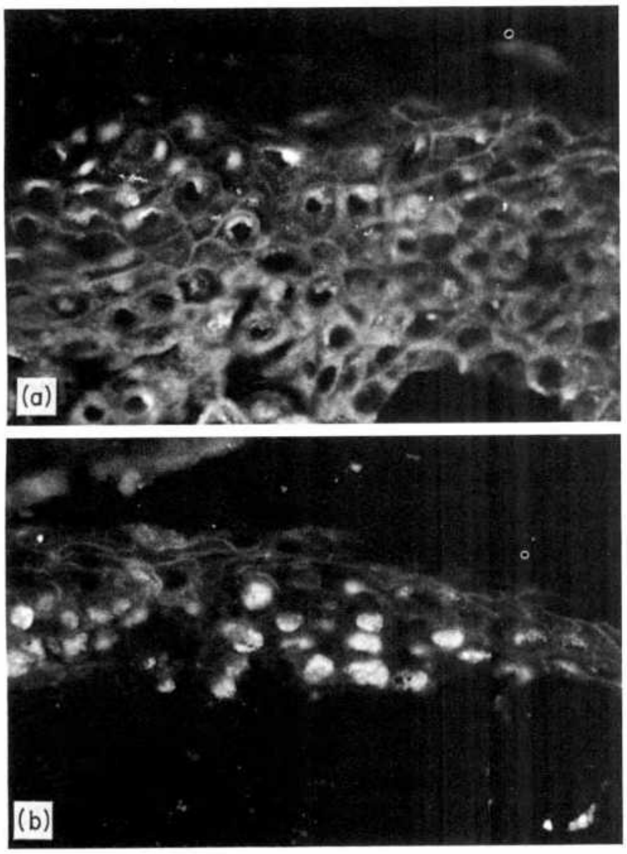

Figure 4. Roof of Hailey–Hailey lesion. Immunostaining of frozen sections with (a) anti-dg1 and (b) anti-dg2/3. There was prominent intracellular staining within the keratinocytes in the relatively cohesive epidermis in the roof of the lesions (× 700).

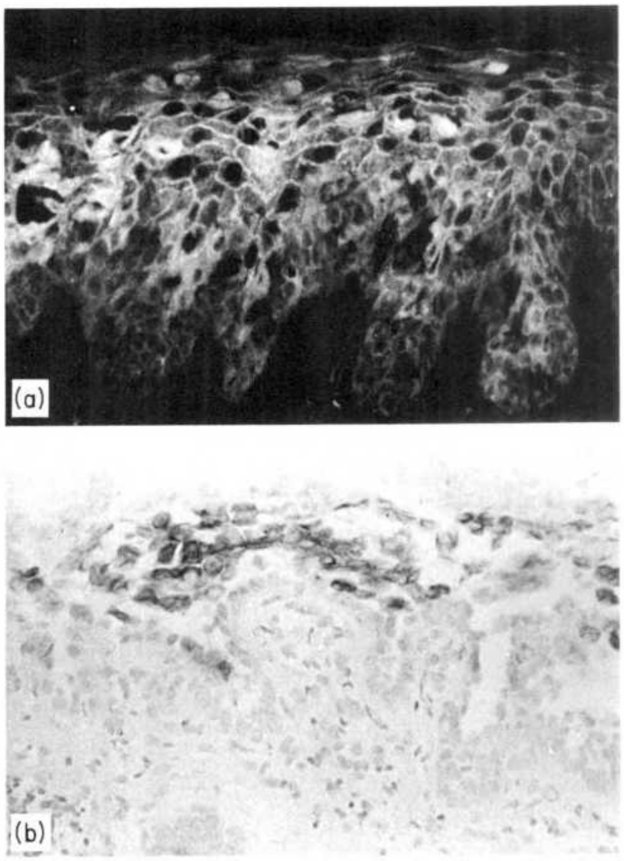

Figure 5. Transient acantholytic dermatosis. (a) Immunostaining of a frozen section with anti-dg 2/3. There is weak staining around keratinocytes in the proliferative basal buds but intense diffuse cytoplasmic staining of many of the cells in the abnormal epidermis (× 400). (b) Immunostaining of paraffin section with anti-dg1. The staining pattern was essentially similar to that obtained in Darier's disease with minimal staining in the basal buds but intense cytoplasmic staining within the majority of the acantholytic cells (× 400).

prominent intracellular staining within the keratinocytes in the relatively cohesive epidermis in the roof of the lesions (Fig. 3c, 4a and b).

Transient acantholytic dermatosis

Anti-dp1/2 (11-5F), anti-dg1 (33-3D and 32-2B) and anti-dg2/3 (52-3D). There was a normal pattern of staining in the perilesional skin. In the lesional skin the staining patterns with the antibodies were similar to those obtained in Darier's disease (Fig. 5).

Suction blisters

Cytoplasmic staining was present within the majority of the acantholytic cells but it was of interest to determine whether acantholysis preceded the development of cytoplasmic staining. The suction-blister technique was used to induce fresh acantholysis. In Darier's disease, although suction does not induce intraepidermal cleavage in normal skin, it accentuates intraepidermal cleavage and acantholysis in lesional skin.[24] In contrast, in Hailey–Hailey disease suction causes intraepidermal cleavage with acantholysis in skin that is clinically and histologically normal.[23]

(a) *Normal skin: frozen material (one blister)*

Anti-dp1/2 (11-5F), anti-dg1 (33-3D) and anti-dg2/3 (52-3D). There was a normal peripheral pattern of staining around the keratinocytes in the intact epidermis forming the roof of the suction blister.

(b) *Darier's disease: frozen material (two blisters in lesions)*

Anti-dp1/2 (11-5F), anti-dg1 (33-3D) and anti-dg2/3 (52-3D). There was intense diffuse staining of the majority of the acantholytic cells in the lesion, but there

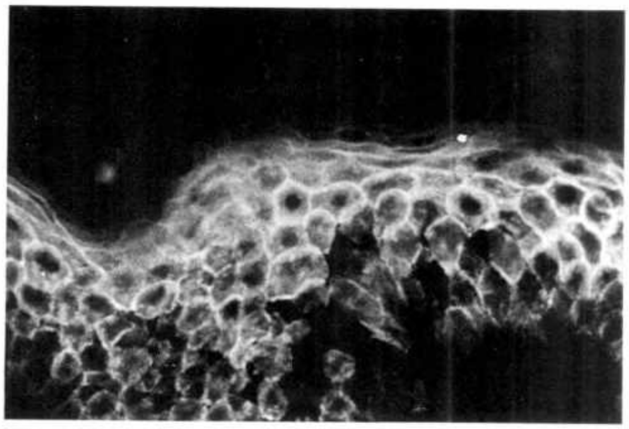

Figure 6. Suction blister on clinically uninvolved forearm skin in Hailey–Hailey disease. Immunostaining of a frozen section with anti-dg1. Suction caused intraepidermal cleavage and this was associated with some reduction in the intensity of peripheral staining around the separating keratinocytes but there was no cytoplasmic staining within these cells (× 400).

were some unstained or very weakly stained acantholytic cells near the base of the blister.

(c) *Hailey–Hailey disease; frozen material (one blister on clinically uninvolved skin)*

Anti-dp1/2 (11-5F), anti-dg1 (33-3D) and anti-dg2/3 (52-3D). Intraepidermal cleavage was associated with fragmentation and reduction in peripheral staining around the separating keratinocytes, but there was no cytoplasmic staining in these cells (Fig. 6).

Triton-X 100 Extraction

The presence of diffuse cytoplasmic staining for desmosomal components in acantholytic cells might indicate that these are soluble components unattached to the cytoskeletal filaments. In order to test this, frozen sections were extracted for 45 min with 0·5% Triton-X 100 before staining with desmosomal antibodies.

(a) *Normal skin*

Anti-dp1/2 (11-5F), anti-dg1 (33-3D) and anti-dg2/3 (52-3D). There was a slight reduction in the intensity of the peripheral staining around the keratinocytes.

(b) *Darier's disease and transient acantholytic dermatosis (TAD)*

Anti-dp1/2 (11-5F). There was very little decrease in the intensity of the staining of the acantholytic cells.

Anti-dg1 (33-3D) and anti-dg2/3 (52-3D). Some of the diffuse staining was removed but it was possible to detect focal areas of intense intracellular staining.

(c) *Hailey–Hailey disease*

Anti-dp1/2 (11-5F). As in Darier's disease the intensity of the staining in the acantholytic cells was only slightly reduced.

Anti-dg1 (33-3D) and anti-dg2/3 (52-3D). In the perilesional skin, the peripheral staining was retained. In the lesion, the diffuse fluorescence in the acantholytic cells was reduced but focal intracellular stain was retained within the acantholytic cells and some cells in the roof of the lesion.

Discussion

These studies have demonstrated that the major desmosomal proteins and glycoproteins are present in Darier's disease and Hailey–Hailey disease. However, there are striking alterations in the pattern of staining for desmosomal components within the acantholytic keratinocytes. In normal keratinocytes staining for desmosomal components is localized to the cell periphery but in the acantholytic cells there were various patterns of cytoplasmic staining. In the majority of acantholytic cells in Darier's disease dps and dgs were distributed in a ring around the nucleus or diffusely in the cytoplasm. Occasionally it was possible to detect focal perinuclear accumulation of dgs. The findings in the acquired disorder, TAD, were similar with some acantholytic cells showing cytoplasmic staining for desmosomal components. In the acantholytic cells of Hailey–Hailey disease dps showed a diffuse distribution with a punctate background, but there were also abnormal clumps of peripheral stain around the basal cells. Dgs were distributed diffusely but with striking focal perinuclear accumulations. There was also bright focal perinuclear cytoplasmic staining within the keratinocytes of the epidermal roof.

The most likely explanation for cytoplasmic desmosomal staining in acantholytic cells is that it represents internalization of half desmosomes associated with loss of intercellular adhesion. In experimental studies internalization of desmosomes halves has been shown to follow cellular dissociation by proteolytic enzymes, reduction in extracellular calcium concentration or calcium chelation.[27-30] In these studies internalized desmosomal halves are associated with membranous vacuoles and remain attached to keratin filaments. In cultured keratinocytes, loss of adhesion gives rise to intense diffuse cytoplasmic staining for desmosomal components[31] similar to the pattern seen in the present study. This staining pattern may arise because the cells possess a large number of desmosomes which give rise to apparently diffuse staining when they become concentrated in the cytoplasm. In cells with fewer desmosomes (MDCK cells) desmosomal internalization gives rise initially to a narrow ring of stain which gradually (3 h) contracts into a small number of focal accumulations near the nucleus.[28]

In the present study, treatment of sections with the non-ionic detergent Triton X-100 did not usually remove much of the dp staining, suggesting that the majority of internalized material was associated with the cytoskeleton. Extraction of diffuse dg staining by this method may indicate partial degradation of dgs, releasing them from their cytoskeletal attachments. An alternative explanation for the staining might be that the diffuse material represents a soluble cytoplasmic pool of newly synthesized desmosomal material[32,33] but this seems unlikely as there is no protein synthesis in the cells after acantholysis.[34]

Intracytoplasmic desmosome-like structures have been observed in a number of situations where there is desmosomal splitting including keratin-layer-stripped epidermis,[35] Bowen's disease[36] and squamous cell carcinoma.[37] In Darier's disease and Hailey-Hailey disease investigators have stated that cell separation is associated with half desmosomes persisting on the cell surface and eventual desmosome loss.[2–9] One previous study of Darier's disease emphasized the appearance of intracytoplasmic desmosomal material closely associated with the abnormal keratinisation.[38] In an attempt to account for the cytoplasmic staining for desmosomal components which we have found in these diseases, we have initiated an immunoelectron microscope study of cells within lesions. Preliminary observations (J.L.Holton, S.M.Burge, D.R.Garrod, unpublished) suggest that staining is associated with cytoplasmic intermediate filaments as would be expected from desmosomal internalization. As the keratin filaments in acantholytic cells are clumped around the nucleus, association of desmosomal components with the filaments would account for the staining patterns we have found.

The demonstration that plasminogen is associated with acantholytic cells in Darier's disease[39] suggests that proteolysis of intercellular material may be occurring, leading to desmosomal breakdown and internalization. It is pertinent that experimentally induced breakdown of intermediate filaments does not lead to desmosomal breakdown or loss of intercellular adhesion,[40,41] therefore there is no reason to suppose that the dyskeratosis observed in Darier's disease and Hailey-Hailey disease is the primary cause of acantholysis. In the suction blisters in lesions of Darier's disease there were some acantholytic cells near the base of the lesion which did not show cytoplasmic staining and in those in clinically uninvolved skin in Hailey-Hailey disease, although there was some reduction in peripheral staining around the separating keratinocytes there was no cytoplasmic staining. These observations suggest that the abnormal staining occurs as a consequence of acantholysis.

An important observation is that the distribution of desmosomal components in Hailey-Hailey disease differs in some respects from that in Darier's disease. In Hailey-Hailey disease focal intracellular expression of the antibodies to dg1 and dg2/3 was immediately apparent in the acantholytic cells and but this was much less pronounced in the acantholytic cells in Darier's disease. The abnormal staining in Darier's disease was limited to the acantholytic cells. In contrast, in Hailey-Hailey disease there was also abnormal expression of dps in the basal cell layer beneath the lesion and intracellular staining for dgs in the keratinocytes forming the apparently intact blister roof. These findings are in keeping with the more widespread acantholysis and 'dilapidated brick-wall histology' which is characteristic of the Hailey-Hailey lesion. The differences indicate that the pathogenesis of the diseases differs and confirms that, although there is clinical and histological overlap between the diseases, they are distinct entities. There may be some pathogenic overlap between Darier's disease and TAD. Both may be exacerbated by sun exposure and the histological features and pattern of staining for desmosomal components in the acantholytic cells are similar, but it is not clear how ultraviolet light triggers acantholysis.

Our results support the view that the number and composition of desmosomes changes with keratinocyte differentiation.[19,42] The dps and dgs were expressed less strongly between the lateral surfaces of basal cells than between the apical surface and suprabasal keratinocytes. Differences in the expression of the dgs were more marked than those of the dps. The expression of dgs at the cell membrane increased in intensity from the apical surface of the basal cells to the mid-spinous layer and intense staining persisted to the granular layer. These results confirm those of Skerrow *et al.*[42] who stated that the extent of the rise in available antigen between the lateral and apical surfaces of the basal cell is greater for dg1 than dp3. Differences in the size, frequency and structure of desmosomes present at the lateral surfaces of the basal cell as well as the distribution of non-desmosomal surface glycoproteins may have a role in facilitating cell division, migration or invasion. The nature of the cell-cell contact may regulate the type of desmosome formed and desmosomes may be more labile between basal cells than suprabasal cells.

In Darier's disease, Hailey–Hailey disease and TAD there was very weak expression of the desmosomal components in the basal buds underlying the lesions. This staining pattern is similar to that of the basal cell compartment of normal skin and probably reflects the small size and low frequency of desmosomes between the cells of the buds. There is evidence from previous work that hyperproliferation is a feature of Darier's disease[43,44] and less intense staining between the basal cells may reflect the proliferative capacity of the cells in the basal buds.

The mechanisms which regulate desmosome formation and breakdown in normal skin, thereby facilitating the proliferation and upward movement of keratinocytes, are poorly understood. There is variation in the sensitivity of desmosomes to splitting agents and desmosomes formed by basal cells are preferentially split by plasmin.[45,46] It seems likely that in the basal buds the desmosomes, which have the staining characteristics of basal cell desmosomes, would also be sensitive to the action of plasmin. In normal skin plasminogen is detected in association with the basal cells[39,47] and focal activation of plasminogen may play a role in the breakdown of desmosomes between basal cells. In Darier's disease there is diffuse expression of plasminogen throughout the proliferative basal buds and in association with the acantholytic cell.[39] Plasminogen is also detected in the basal buds and acantholytic cells in TAC and Hailey–Hailey (S.M.Burge, unpublished observations). It is possible that activation of plasminogen with breakdown of the desmosomes contributes to the cell proliferation and acantholysis seen in these conditions.

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Cutaneous and subcutaneous leiomyosarcoma: a clinicopathological review of 14 cases with reference to antidesmin staining and nuclear DNA patterns studied by flow cytometry

G.F.OLIVER, H.M.REIMAN,* N.J.GONCHOROFF,† S.A.MULLER AND I.J.UMBERT

*Department of Dermatology, *Division of Pathology and †Division of Laboratory Medicine and Pathology, Mayo Clinic and Mayo Foundation, Rochester, MN, U.S.A.*

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Summary

Among 14 patients (11 female and three male) with cutaneous and subcutaneous leiomyosarcomas, metastatic spread occurred in six of the 13 patients with adequate follow-up. The tumours were classified with regard to tumour grade, number of mitotic figures per 10 high-power fields, reactivity to antidesmin antibodies, and DNA pattern as assessed by flow cytometry. Antidesmin antibody staining was present in only six cases and seemed to be less common in the higher-grade tumours. The DNA pattern was aneuploid in five cases and non-classifiable in one case; all of these cases had metastatic spread. None of the eight remaining diploid cases had metastatic spread. DNA content was a strong predictor of metastatic potential.

Leiomyosarcomas account for about 7% of soft-tissue sarcomas,[1] and superficial leiomyosarcomas account for about 2–3% of all superficial soft-tissue sarcomas.[2] The superficial leiomyosarcomas are divided into intradermal and subcutaneous tumours and can occur at any age, but are most common between the fifth and seventh decades of life.[1]

The largest series, reported by Fields and Helwig,[3] included 65 primary cutaneous leiomyosarcomas and 15 primary superficial subcutaneous leiomyosarcomas. These authors related metastatic potential primarily to tumour depth and to a lesser extent to the degree of mitotic activity.

The histological diagnosis of leiomyosarcoma is based on light microscopic findings, and on immunohistochemistry and electron microscopy for undifferentiated tumours.[1] Antidesmin antibodies have been used to differentiate leiomyosarcoma from other spindle cell or pleomorphic tumours in the skin.[4] Recently, flow cytometric analysis of the DNA content of tumours has been used as an indicator of malignant potential, and is useful in the selection of therapy.[5]

We studied 14 cases of superficial leiomyosarcoma and investigated the clinical course, histology and reactivity of the tumour to antidesmin antibody and the results of flow cytometric analysis.

Methods

The records of all Mayo Clinic patients (from the files of the Division of Pathology and the Department of Dermatology) with a diagnosis of cutaneous leiomyosarcoma from 1965 to February 1988 were reviewed. Cases with a cutaneous or subcutaneous tumour that had the histological features of leiomyosarcoma[1–3] and in which the tissue was still available were included. Histological features that indicated smooth muscle differentiation included elongated or cigar-shaped nuclei within an eosinophilic cytoplasm, cells arranged in fascicles and with perinuclear vacuoles. The diagnosis of malignancy required increased cellularity and nuclear pleomorphism and atypia. These features were subjectively assessed and were of primary importance in assigning the histological grade. In addition, increased mitotic activity and necrosis were also noted. If these findings were present, depending on their degree, the tumour could be assigned a higher histological grade. Case histories were reviewed to confirm the location of the tumour and to obtain demographic and follow-up data.

Sections were obtained from the paraffin-embedded formalin-fixed specimens and stained with hematoxylin and eosin. In addition, antidesmin immunohistochemical staining was performed on other sections. A prediluted mouse antidesmin (Biogenics Laboratories, San Ramon, CA, U.S.A.) was used with a standard two-step

Correspondence: Dr S.A.Muller, Mayo Clinic, 200 First Street SW, Rochester, MN 55905, U.S.A.

immunoperoxidase method as specified by the manufacturer.

Histological specimens were examined for grade of malignancy, number of mitotic figures per 10 high-power fields, and the presence of necrosis by routine light microscopy. In the immunoperoxidase-stained specimens, the presence or absence of antidesmin staining of the tumour was noted, and the presence of a positive internal control was noted if the tumour was negative for antidesmin.

All cases were submitted for flow cytometry analysis of nuclear DNA patterns. Adjacent sections of the tumour from each paraffin block were used for histological analysis and flow cytometry.

Three 50-μM sections were used for flow cytometry analysis to minimize cellular artifacts.[6] The cell nuclei were extracted using a modification of the method of Hedley *et al.*[7] After dewaxing and rehydration, but before sequential treatment with 0·25% trypsin and 0·5% pepsin, the sections were sonicated for 3 min with a Branson model 450 power supply fitted with a model 102 converter and a model 109-067-685 cup horn (Branson Ultrasonics, Danbury, CT, U.S.A.). After enzymatic treatment, the samples were sonicated a second time for 2 min. This sonication procedure has two benefits over the standard procedure: it increases the nuclei yield and it removes cell doublets. The samples were then treated with RNase and stained with propidium iodide.[8] Data from 10,000 cells were collected as list-mode-data files with a fluorescence-activated cell analyser (FACS analyser; Becton Dickinson, Mountain View, CA, U.S.A.).

The DNA content of the tumours was classified as DNA diploid if the sample met the following conditions: (i) it contained two identifiable peaks, both of which had a coefficient of variation (CV) of less than 12%; (ii) the second peak contained less than 15% of the cells; and (iii) the second peak had a DNA index between 1·9 and 2·1 (the DNA index was determined by dividing the mean channel number of the first peak by the mean channel number of the second peak). The DNA content was considered DNA tetraploid if the G2 + M peak contained more than 15% of the cells, and it was DNA non-classifiable if the tumour contained a G0/G1 peak with a wide CV ($\geqslant 12\%$) but no identifiable G2 + M peak. All samples were run in parallel with a normal kidney specimen known to give a consistent DNA diploid pattern and a lung tumour that was consistently DNA aneuploid. The histograms were classified by four independent readers. The CV of the first peak was assumed to be the DNA diploid component and calculated using the Consort 30 software program (Becton Dickinson). The percentage of cells in S-phase was calculated with a rectangular model algorithm developed by Baisch *et al.*[9]

Results

The 14 patients (11 female and three male; aged 13–84 years, mean age 57·8 years) presented between 1967 and 1988; of these, seven cases were diagnosed from 1985 to 1988 (Table 1). The tumour was located on the leg in eight patients, on the trunk in four, on the arm in one, and on the labia in one. The depth of the tumour was determined at the time of operation. These findings were confirmed by examination of histological specimens. Of the 10 patients who had subcutaneous tumours, two (Cases 3 and 7) had extension of the tumour into muscle; of the four patients with dermal tumours, two (Cases 8 and 13) showed microscopic extension of the tumour into the subcutaneous tissue.

All the patients had been referred to the Mayo Clinic. This restriction excluded two patients who were described recently.[10] Ten patients had undergone excision of their tumour before referral. The specimens from the original excision were available for the cases in which wide local excision had been performed, and the data were obtained from these specimens. No definite lymphatic or vascular invasion was noted in the tumours.

All patients presented with a slowly growing tumour that had been present for up to 2 years pre-operatively. One patient (Case 11) had a history of radiotherapy to the site some years before development of the tumour. Treatment consisted of wide local excision in nine cases (five subcutaneous, four dermal), local excision and radiotherapy in two (subcutaneous), *en bloc* excision in two (subcutaneous), and amputation in one (subcutaneous). Local tumour recurrence occurred in five cases (all subcutaneous tumours) after wide excision in three and after *en bloc* excision in two. Metastatic spread was noted in six cases (all subcutaneous) (Table 1).

Histology

All tumours demonstrated histological features characteristic of leiomyosarcoma (Fig. 1). Necrosis was present in six cases (all subcutaneous) and was seen in tumours of histological grade 3 or 4 and in tumours more than 1 cm in diameter. The tumour was grade 2 in four cases, grade 3 in seven cases, and grade 4 in three cases. The number of mitotic figures present was high for all grade 4 tumours and ranged from <1 to 6 per 10 high-power

Table 1. Data from 14 patients with cutaneous leiomyosarcoma

	Patient characteristics					Tumour characteristics					
Case	Age (year)	Sex	Site of tumour	Metastases	Duration of survival (years)	Grade	Mitotic figures/10 h.p.f.	Desmin staining	DNA ploidy pattern	CV of G0/G1 peak	S-phase cells (%)
1	43	F	Knee, S/C	No	>2	3	3	–	Diploid	7·7	8·0
2	57	F	Thigh, S/C	Yes	7*	3	<1	–	Aneuploid	5·7	—
3	76	F	Leg, S/C	Yes	7*	2	0	+	Non-classifiable	—	—
4	58	F	Arm, S/C	Yes	1*	4	18	–	Aneuploid	9·2	—
5	84	F	Labia, S/C	No	>1	2	0	+	Diploid	9·2	16·0
6	57	F	Leg, S/C	Yes	2*	4	19	+	Aneuploid	8·6	—
7	39	F	Chest, S/C	No FU	No FU	3	3	–	Diploid	9·9	26·0
8	13	M	Shoulder, dermal	No	>6 months	3	1	–	Diploid	9·6	13·0
9	67	M	Calf, dermal	No	>2	2	2	+	Diploid	10·5	18·0
10	51	F	Back, dermal	No	>3	3	3	–	Diploid	8·5	22·0
11	80	F	Shoulder, S/C	Yes	2*	3	6	–	Aneuploid	6·7	—
12	70	M	Leg, S/C	No	>7	3	<1	+	Diploid	9·0	20·0
13	52	F	Thigh, dermal	No	>3	2	0	+	Diploid	7·8	9·0
14	62	F	Thigh, S/C	Yes	>1	4	7	–	Aneuploid	9·2	—
	Mean 57·8									Mean 8·6 SD_{n-1} 1·3	Mean 18·9 SD_{n-1} 9·3

* Deceased patients.

Abbreviations: CV, coefficient of variation; FU, follow-up; h.p.f., high-power field; S/C, subcutaneous; +, positive; –, negative.

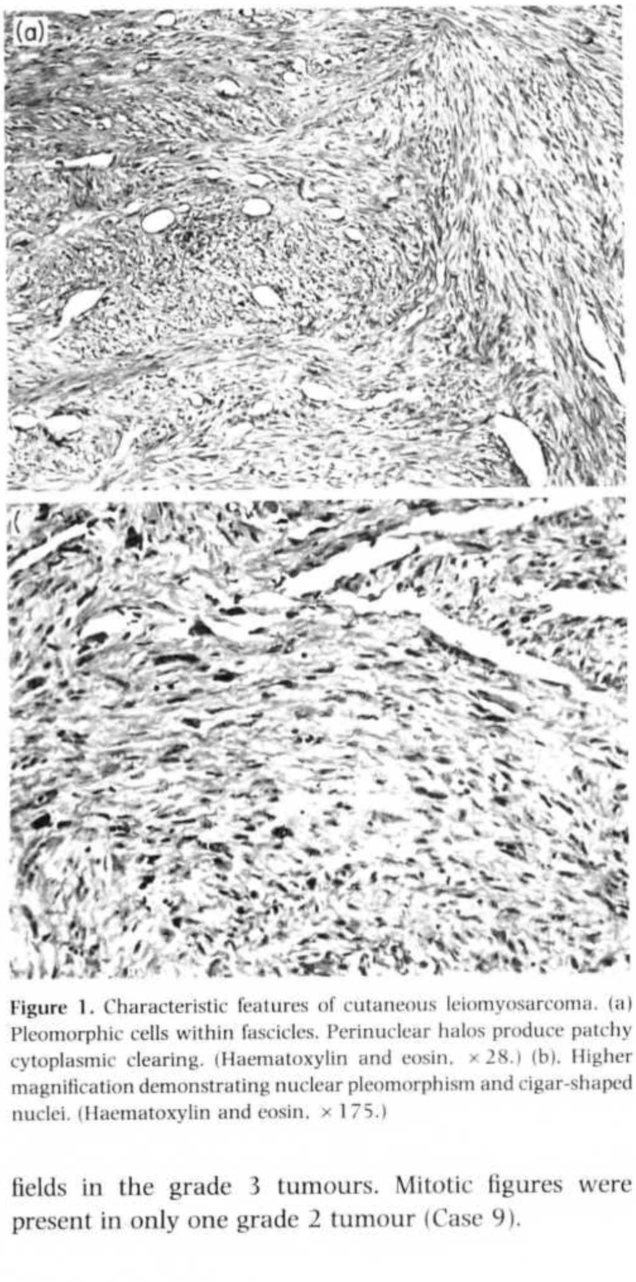

Figure 1. Characteristic features of cutaneous leiomyosarcoma. (a) Pleomorphic cells within fascicles. Perinuclear halos produce patchy cytoplasmic clearing. (Haematoxylin and eosin, × 28.) (b). Higher magnification demonstrating nuclear pleomorphism and cigar-shaped nuclei. (Haematoxylin and eosin, × 175.)

fields in the grade 3 tumours. Mitotic figures were present in only one grade 2 tumour (Case 9).

Antidesmin staining

Only six of the 14 tumours were positive with antidesmin staining; of the eight negative cases, four contained positive-staining skeletal muscle as a positive internal control. All grade 2 tumours showed positive staining. Positive staining was present in one of the three grade 4 tumours (Case 6).

Flow cytometry

Results were obtained for all specimens submitted for flow cytometric analysis of DNA content. The flow cytometry pattern in Case 3 was DNA non-classifiable. Of the 13 classifiable cases, five demonstrated DNA aneuploidy, one had a high percentage of cells in the S-phase with a DNA diploid pattern (Case 7 had 26·0% S-phase cells), and seven were DNA diploid and had a low S-phase percentage (Fig. 2).

Four of the five cases with a DNA aneuploid pattern had metastatic spread of the tumour to the lung. The fifth patient with a DNA aneuploid pattern had metastatic leiomyosarcoma in a lymph node; she died soon after the tumour was excised, and no autopsy was performed. The only other patient to experience metastatic tumour spread was Case 3 (DNA non-classifiable). None of the four dermal tumours were DNA aneuploid and none had metastatic spread. Of the 10 subcutaneous tumours, four cases were DNA diploid and without metastases.

Although the sample size is small, within the DNA diploid cases the number of mitotic cells correlated with the percentage of S-phase cells as determined by flow cytometry (Table 1; rank correlation coefficient, 0·48).

Table 2. Correlation of ploidy pattern with histologic grade in 14 patients with cutaneous leiomyosarcoma

DNA ploidy pattern	Tumour grade 1	2	3	4
Diploid	—	0/3*	0/4	—
Diploid, high S-phase	—	—	0/1	—
Aneuploid	—	—	2/2	3/3
Non-classifiable	—	1/1	—	—

* Number of patients with metastasis/total number.

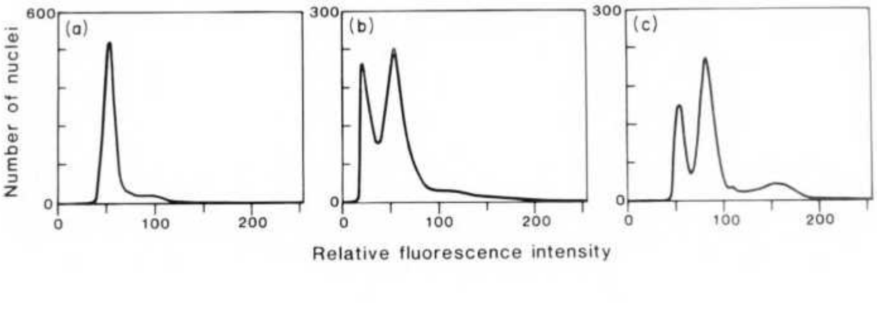

Figure 2. Representative flow cytometry histograms from patients with cutaneous leiomyosarcoma. (a) DNA diploid pattern (Case 5); (b) DNA nonclassifiable (Case 3); (c) DNA aneuploid pattern (Case 6).

Additionally, the histological grade correlated with ploidy (Table 2). Of the three grade 4 tumours, two were aneuploid and one was diploid with high S-phase.

Discussion

Leiomyosarcoma of the skin and subcutaneous tissue is routinely diagnosed by examination of histological specimens stained with hematoxylin and eosin.[1-3,11] The implications of this diagnosis and an assessment of the risk of metastatic spread are important in determining therapy and prognosis. Monoclonal antibodies have been used to identify the predominant cell types of tumours and inflammatory diseases. Within solid tumours, the antibodies to intermediate filaments (keratin, vimentin, desmin, neurofilament, glial fibrillary acidic protein) have been proposed as reliable markers for tumour classification.[12] In leiomyosarcomas antidesmin antibodies have been found to be positive in 47–85% of cases.[11-13] In our series, reactivity with the antidesmin antibody was found in only six (43%) of the 14 cases. A negative result was more common in the higher-grade tumours. The presence or absence of antidesmin staining did not relate to the risk of metastatic spread.

The finding of antidesmin antibody staining in undifferentiated spindle-cell neoplasms is helpful, although it is not diagnostic of a smooth-muscle-derived tumour.[4] Conversely, the absence of antidesmin antibody staining does not exclude such a tumour[4] and in our experience is not uncommon. For those rare tumours in which routine histology and immunostaining fail to provide a classification of the tumour type, electron microscopy offers another means for diagnosis.[13-14]

Fields and Helwig[3] found that metastatic spread occurred in 33% of leiomyosarcomas located in the subcutaneous tissue. However, none of their cases of dermal tumours had metastatic spread. They concluded that the depth of the primary tumour was the major factor for metastatic spread. The mitotic rate of the tumour also provided some predictive information in their series. They were unable to explain why some tumours metastasized while others did not despite similar tumour characteristics and size.

Local recurrence of tumour occurs in about 40% of cutaneous leiomyosarcomas and in about 50% of subcutaneous leiomyosarcomas.[1,3] The recurrences seem related to the adequacy of initial excision. Five of our 10 subcutaneous cases and one of our four dermal cases had local recurrence. In our series, local recurrence was more common in the tumours with a DNA aneuploid pattern, but two cases had metastases without local recurrence, and two cases with recurrence had no metastatic spread.

Flow cytometry to study DNA content has proved to be a method for detecting gross cytogenetic abnormalities and for measuring cell cycle fractions. It has been used in solid tumours to provide an index of malignant behaviour.[5,15-17] DNA aneuploidy is positively correlated with increased mortality and local recurrence.[18-20]

Two recent studies examined the DNA content of leiomyosarcomas of the stomach and uterus. In the study by Tsushima *et al.*[21] a DNA aneuploid pattern was present in 23% of 44 leiomyosarcomas of the stomach and in 10% of leiomyomas and leiomyoblastomas. The authors found that DNA aneuploidy within the leiomyosarcoma group was significantly correlated with survival, as were tumour grade and tumour size. Ploidy could not be used for definite differentiation of malignant from benign gastric smooth-muscle tumours, but it did confer prognostic information. Our studies confirm these observations. Tsushima *et al.*[22] studied DNA ploidy patterns in benign and malignant smooth muscle tumours of the uterus. The nuclear DNA patterns could not distinguish benign from malignant tumours, but they did correlate with tumour grade, size, and mitotic rate. DNA aneuploidy also conferred some additional prognostic significance in uterine leiomyosarcomas.

In our series, all DNA aneuploid cases had metastatic disease. One additional patient also had metastatic disease (Case 3). In this patient, the DNA histogram was not classifiable by our established criteria because it had a wide G0/G1 peak and no identifiable G2 + M peak. This histogram probably represents a tumour with an abnormal DNA content.

We also measured the S-phase percentage in the DNA diploid cases. This percentage did correlate with the number of mitotic figures. Although S-phase was not a significant factor in patient survival, in other tumour systems, notably breast cancer[23,24] and multiple myeloma,[25] a high percentage of cells in S-phase is correlated with a poor prognosis.

In our series of patients, metastatic spread of the tumour was related to DNA ploidy. High tumour grade and mitotic rate correlated with a DNA aneuploid pattern and a high incidence of metastatic spread. For the grade 3 tumours, DNA ploidy was the strongest predictor of metastatic potential.

The 14 cases of cutaneous and subcutaneous leiomyosarcomas demonstrated the typical clinical and histopathological features of this rare superficial soft tissue sarcoma. We have found that immunohistochemistry with antidesmin antibody is generally not useful as a diagnostic aid in these tumours. The risk of local recurrence seems to be related to the adequacy of excision, whereas metastatic spread is related to depth, histological grade of the tumour, and the DNA content as assessed by flow cytometry. Tumours with a non-DNA diploid pattern seem to be associated with a worse prognosis.

Acknowledgments

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Sunscreen protection against UVB, UVA and blue light: an *in vivo* and *in vitro* comparison

B.L.DIFFEY AND P.M.FARR*

*Regional Medical Physics Department, Dryburn Hospital, Durham, *Department of Dermatology, Royal Victoria Infirmary, Newcastle upon Tyne, U.K.*

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Summary The photoprotection against UVB, UVA and blue light provided by a widely prescribed sunscreen (RoC 15^+ A+B) was compared with two new products containing microfine titanium dioxide (Sun E45 lotion SPF 15 and Sun E45 cream SPF 20). Comparisons were made *in vivo* using photosensitive patients with either chronic actinic dermatitis or erythropoietic protoporphyria, and *in vitro* using a newly developed spectrophotometric assay. Good agreement was obtained between the *in-vivo* and *in-vitro* methods at each waveband. All products showed high protection against UVB radiation, but the products containing microfine titanium dioxide showed significantly higher protection against both UVA and blue light than RoC 15^+ A+B. Products containing microfine titanium dioxide are likely to offer superior photoprotection in those patients who are abnormally sensitive to long wavelength ultraviolet radiation than products which are currently available on prescription.

A large number of high-protection-factor sunscreens are available for use by patients with photosensitive skin disorders. The sun protection factor (SPF) of these products refers largely to protection against UVB radiation (290–320 nm), whereas most idiopathic photosensitivity disorders, such as polymorphic light eruption (PLE) and chronic actinic dermatitis (CAD), and drug-induced photosensitivity, require protection against UVA (320–400 nm).[1-3] The UVA protection factor of sunscreens is difficult to test *in vivo* because of the relative erythemal insensitivity of normal skin to this waveband. A new *in vitro* technique for measurement of sunscreen protection at any wavelength has been described recently.[4] Results of sunscreen testing using this technique have shown good agreement with the SPF values claimed by manufacturers, and those of reference formulations defined by American and German standards authorities (FDA and DIN). When tested using the *in-vitro* technique a range of products was shown to give poor protection against UVA, and even so called 'total sunblocks' were found to have protection factors of 5 or less at wavelengths greater than 350 nm.[4]

In this study we have compared the protection acheived with a widely prescribed topical sunscreen with that of newly developed products based on microfine titanium dioxide. The testing of photosensitive patients allowed the *in-vivo* measurement of protection to UVA and visible light. The results were used to validate further the *in-vitro* technique[4] for measurement of sunscreen protection.

Methods

Sunscreens

Three products were compared.

1. RoC opaque total sunblock cream 15^+ A+B (Laboratories RoC (U.K.) Ltd., London). Active ingredients: cinnamic ester, mineral pigments.
2. Sun E45 lotion SPF 15 (Crookes Health Care, Nottingham, U.K.). Active ingredient: microfine titanium dioxide (13·4% concentration).
3. Sun E45 cream SPF 20 (Crookes Health Care, Nottingham). Active ingredient: microfine titanium dioxide (18% concentration).

Patients

Two groups of patients were studied. The first group comprised 11 patients with severe chronic actinic dermatitis (CAD) who were known to be abnormally sensitive to UVA radiation. The second group consisted of seven male patients with erythropoietic protoporphyria (EPP).

In-vivo photoirradiation

The 11 patients with CAD were irradiated using an

Correspondence: Dr B.L.Diffey, Regional Medical Physics Department, Dryburn Hospital, Durham DH1 5TW, U.K.

irradiation monochromator[5] with three different spectral regions at central wavelengths (±full bandwidth at half maximal intensity) of 300±5, 350±30 and 400±30 nm, respectively. A Schott colour glass filter (WG335) was inserted into the radiation beam at 350 and 400 nm to eliminate any stray UVB radiation.

For each waveband a series of sites on the mid-back was exposed using an irradiation field of 20 mm^2 and a geometric progression of $\sqrt{2}$ for the dose increments. The lowest dose (MED) at each waveband which produced minimally perceptible erythema 24 h after irradiation was noted. A 50 cm^2 area was outlined on either side of the back as close as possible to the previously irradiated sites and RoC sunblock cream or Sun E45 lotion (100 μl) were applied randomly to either side of the back and spread as uniformly as possible with a gloved finger, resulting in a surface density of 2 μl/cm^2. The products were allowed to dry for 15 min before multiples of the MEDs on unprotected skin were given (Table 1). After a further 24 h, the MEDs in each spectral region were noted, and protection factors calculated for the two sunscreens in each waveband by dividing the MED on protected skin by the MED on unprotected skin.

The EPP patients were irradiated with blue light using a 1 kW high-pressure mercury lamp doped with iron and gallium iodides to enhance the spectral emission in the range 350–450 nm. The radiation passed through optical filters to isolate blue light (400–470 nm; Fig. 1) and was focused into a light guide to produce a uniform beam (10 mm diameter) on the skin surface with an irradiance of 130 mW/cm^2.

Five sites, 10 mm in diameter, were marked on one side of the back of each patient. The basal erythema was measured three times at each site using a reflectance instrument.[6] The sites were then irradiated consecutively using a geometrical series of increasing exposures (dose increment factor 2) with a maximum exposure dose of 48 J/cm^2. One hour after irradiation the measurements of erythema at each of the sites were repeated and the increase in erythema (ΔE) expressed as the difference between the pre- and post-irradiation indices.[7] RoC sunblock cream and Sun E45 cream were applied at a surface density of 2 μl/cm^2 to the back, spread uniformly with a gloved finger, and allowed to dry for 15 min.

Six sites were irradiated (maximum dose 96 J/cm^2) on the skin protected by each sunscreen and the erythema measurements made before and 1 h after irradiation.

In-vitro determination of protection factors

The protection against wavelengths of UVR was determined using a recently developed assay.[4] Briefly, the spectral transmission of ultraviolet radiation through a sample of Transpore™ tape was measured with and without the applied sunscreen. The products were applied at a surface density of 2 μl/cm^2 in both the *in-vitro* and *in-vivo* studies.

Results

Chronic actinic dermatitis patients

The minimal erythema doses (MEDs) determined on the unprotected skin of each patient are listed in Table 2, and the protection factors given in Table 3. The median protection factors for each sunscreen in the three wavebands were determined using a life-table method.[8] Also shown in Table 3 are the protection factors for each waveband derived from the *in-vitro* assay. To make a direct comparison it was necessary to combine the monochromatic protection factors determined *in vitro* (Table 4) with the spectral emission from the irradiation monochromator and the erythema action spectum. RoC sunblock cream provided significantly higher protection at 300 nm than the Sun 45 lotion ($P<0{\cdot}01$; logrank test), although the reverse was the case at 350 and 400 nm ($P<0{\cdot}01$).

Erythropoietic protoporphyria patients

The increase in erythema indices (ΔE) as measured on the unprotected skin sites was plotted against the logarithm of the blue-light dose for each patient. This was repeated for the sites protected with RoC sunblock

Table 1. Multiples of the minimal erythema dose on unprotected skin given to sunscreen-protected skin

Waveband (nm)	Sun E45 lotion					RoC 15$^+$ A+B				
300±5	19	24	30	38	48	32	40	50	63	80
350±30	10	13	16	20	25	4	5	6	8	10
400±30	2	3	4	5	6	1·3	1·6	2	2·5	3

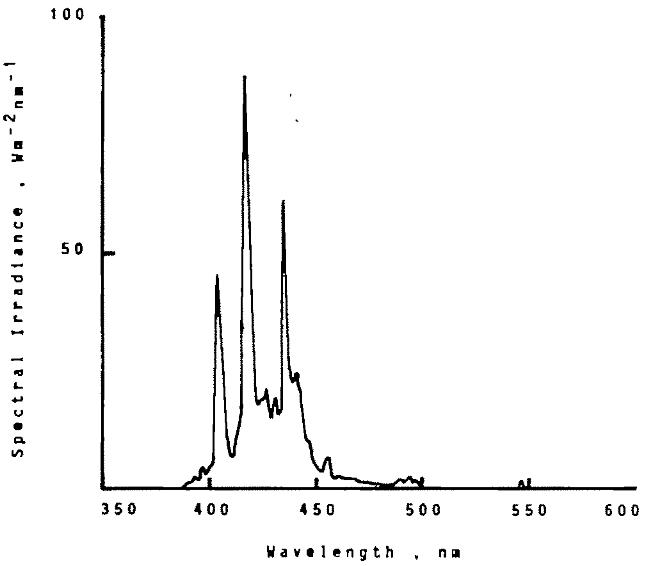

Figure 1. Spectral irradiance from the blue-light source in the plane of the patient's skin

Table 2. Minimal erythema doses on the unprotected skin of patients with chronic actinic dermatitis

			Minimal erythema dose		
Patient	Sex	Age	300±5 nm (mJ/cm²)	350±30 nm (J/cm²)	400±30 nm (J/cm²)
1	F	60	28	7	40
2	M	55	20	7	40
3	M	67	20	10	20
4	F	52	10	1·8	28
5	M	59	5	1·8	20
6	F	39	7	2·5	14
7	F	66	0·9	5	14
8	M	57	5	5	20
9	M	59	0·6	0·9	10
10	M	77	0·6	0·6	7
11	M	39	14	7	40

cream and Sun E45 cream. An example of the responses obtained in one patient is shown in Figure 2. The solid lines in Figure 2 are the regression curves obtained by expressing ΔE as a logit function of the logarithm of dose. By this process the doses of blue light that would be expected to result in a ΔE of 0·025 (equivalent to a just perceptible redness) on unprotected and sunscreen-protected sites were interpolated. These doses, termed $D_{0\cdot025}$, are listed in Table 5. The ratio of $D_{0\cdot025}$ on unprotected skin to that on sunscreen-protected skin gives a measure of the protection against blue light in patients with EPP. It may be seen (Table 5) that RoC sunblock cream provides no protection, whilst the Sun E45 cream provides a protection factor of 2–3. This difference in protection is significant ($P<0\cdot02$; logrank test).

In-vitro assay

The monochromatic protection factors at 5-nm intervals from 290 to 400 nm for each product are given in Table 4.

The sun protection factors (SPF) given at the bottom of Table 4 were predicted from the monochromatic protection factors at each wavelength [PF(λ)] according to:[4]

$$\mathrm{SPF} = \sum_{290}^{400} E(\lambda)\varepsilon(\lambda) \Big/ \sum_{290}^{400} E(\lambda)\varepsilon(\lambda)/\mathrm{PF}(\lambda) \qquad (1)$$

where E(λ) is the spectral irradiance of terrestrial sunlight under defined conditions and ε(λ) is the relative effectiveness of UVR at wavelength λ nm in producing delayed erythema in human skin (so-called 'erythema action spectrum').

Values of E(λ) used here were chosen to represent midday midsummer sunlight for southern Europe (latitude 40°N; solar zenith angle 20°; ozone layer thickness 0·305 cm), and were derived from published data.[9,10] The erythema action spectrum used in the calculation was that recently adopted by the International Commission on Illumination (CIE) as a reference action spectrum.[11]

Discussion

We have measured the protection factors of a widely prescribed, commercially available sunscreen and two formulations of a newly developed product based on microfine titanium dioxide. All the products tested showed high protection at 300 nm (UVB). The median protection factors at 350 nm (UVA) and 400–470 nm (blue light) were significantly higher for the titanium dioxide sunscreen than for the commercially available product (RoC 15$^+$ A+B).

The use of photosensitive patients with CAD as test subjects allowed the *in-vivo* measurement of protection factors at 350 and 400 nm. The relative erythemal insensitivity of the skin at these wavelengths precludes the use of normal subjects for sunscreen testing because of the long irradiation times that would be required to achieve erythema with conventional light sources. To overcome this problem others have sensitized the skin with psoralens.[12-14] However, this technique creates an

Table 3. Protection factors obtained in the sunscreen protected skin of patients with chronic actinic dermatitis

	Sun E45 lotion			RoC 15$^+$ A+B		
Patient	300±5	350±30	400±30	300±5	350±30	400±30
1	30	16	3	50	5	2
2	24	16	3	63	6	1·6
3	38	16	ND	63	5	ND
4	30	13	2	50	8	1·3
5	24	13	3	50	4	2·5
6	38	20	4	63	8	3
7	24	10	3	63	5	1·6
8	38	13	2	>63	8	2
9	38	16	3	80	6	1·6
10	48	20	2	>80	10	1·3
11	19	16	3	50	10	1·6
Median	29	14	2·7	55	5·8	1·5
90% confidence interval	11–43	9–19	1·7–3·5	49–>80	2·5–10	1·1–2·7
Derived from *in-vitro* assay	24	14	4·2	49	6·2	3·0

ND, not done.

Table 4. Monochromatic and sun protection factors determined by *in-vitro* assay

	Monochromatic protection factor		
Wavelength (nm)	Sun E45 lotion	Sun E45 cream	RoC 15$^+$ A+B
290	23	32	43
295	24	33	47
300	24	33	49
305	24	33	50
310	24	33	51
315	24	33	50
320	24	33	45
325	23	32	35
330	22	31	21
335	20	29	10
340	18	27	6·2
345	15	25	4·8
350	13	22	4·3
355	11	19	4·2
360	9·7	16	4·2
365	8·4	14	4·4
370	7·3	12	4·5
375	6·3	10	4·7
380	5·5	8·4	4·1
385	4·8	6·9	3·3
390	4·2	5·7	3·1
395	3·7	4·8	3·1
400	3·4	4·2	3·0
405	3·2	3·9	3·0
410	3·0	3·7	3·0
415	2·9	3·5	2·9
420	2·8	3·4	2·9
425	2·8	3·3	2·9
430	2·7	3·2	2·9
SPF	20	30	25

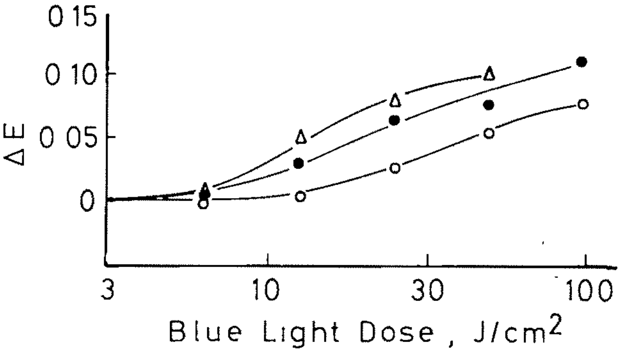

Figure 2. The increase in erythema index (ΔE) measured 1 h after irradiation with different doses of blue light in one patient. △——△, unprotected; ●——●, RoC 15$^+$ A+B; ○——○, Sun E45 cream.

artificial state of photosensitivity and results in an action spectrum for erythema which is biased towards the shorter UVA wavelengths and results in an overestimate of the UVA protection that would be expected in normal skin.[15]

We have described recently the abnormal response of patients with EPP to irradiation with blue light[16] and this response has been used to provide the first *in-vivo* assessment of sunscreen protection at this waveband. In patients with EPP exposed to blue light, after resolution of the flare response, the intensity of the persistent erythema localized to the irradiation sites is dose-dependent.[16] Dose–response curves constructed for this response using an objective measurement of erythema were used to calculate the protection factors of the sunscreens. The median protection factor for the

Table 5. The dose of blue light ($D_{0\,025}$) required to produce a just perceptible erythema one hour after irradiation on unprotected and sunscreen protected skin in patients with EPP

	$D_{0\,025}$, J/cm^2			Protection factor	
Patient	Unprotected skin	Sun E45 cream	RoC 15$^+$ A+B	Sun E45 cream	RoC 15$^+$ A+B
1	21	37	18	1·8	0·86
2	9	24	10	2·7	1 1
3	7	30	7	4·3	1·0
4	17	37	45	2·2	2·6
5	12	43	19	3·6	1 6
6	19	42	16	2·2	0·84
7	12	30	13	2·5	1·1
Median				2·4	1·0
90% confidence interval				1·7–4 3	0 8–2·6
Protection factor at 430 nm from *in-vitro* assay				3·2	2·9

titanium dioxide product was 2·4, whereas RoC 15$^+$ A+B provided no protection (Table 5).

The incorporation into sunscreens of chemicals which absorb in the UVA, such as Parasol 1789 and oxybenzone, only results in protection factors of about 4 or less at 350 nm.[4,15] Higher UVA protection can be achieved by incorporating pigments such as zinc oxide or titanium dioxide. The particle sizes commonly employed are in excess of 0·2 μm and the optical scattering properties of these particles result in only moderate UVA protection factors of around 5 coupled with a white appearance on the skin. By reducing the particle size of titanium dioxide to around 0·05 μm (so-called microfine titanium dioxide) the scattering per unit volume increases, particularly in the ultraviolet and blue end of the optical spectrum, and UVA protection factors of 10 or more can now be achieved (Table 3). The strong wavelength dependency of the scattering of light gives a blue undertone effect when these sunscreens are applied to the skin.

We chose to compare the newly developed microfine titanium dioxide products with RoC 15$^+$ A+B as the manufacturers of this sunscreen claim that both ultraviolet A and B rays are blocked and that skin is protected against all the adverse effects of the sun. RoC 15$^+$ A+B is also the most commonly prescribed sunscreen in the U.K. (personal communication, Department of Health, 1989). Our results show that RoC 15$^+$ A+B only achieves a protection factor of approximately 6 at 350 nm (UVA) and provides no protection at all against blue light.

The sunscreen products were also tested using a new *in-vitro* technique that allows measurement of monochromatic protection factors at any wavelength. Results from testing standard reference formulations using this technique have shown good agreement with SPF values determined by American and German standards authorities (FDA and DIN).[4] However, these results refer mainly to protection against UVB wavelengths and the technique has not been validated for UVA or visible light protection. The *in-vitro* measurements (Tables 3 and 5) show good agreement with those obtained by standard *in-vivo* testing especially within the UVA and UVB wavebands, and thus provide further validation that this technique is a reliable method of determining ultraviolet protection.

We have shown that the newly developed sunscreen products based on microfine titanium dioxide provide significantly higher UVA and visible light protection than RoC 15$^+$ A+B. The new products should be of greater benefit than those presently available for photosensitive patients who require UVA protection. Like all pigment-based sunscreens they remain visible when applied to the skin, especially at the recommended surface density of 2 μl/cm^2. However, the method of formulation results in good skin adherence and the products will not rub off onto clothing. Also the microfine nature of the particles of titanium oxide minimizes the intensity of the white appearance of the skin.

Acknowledgments

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Quantification of skin-colour changes induced by topical corticosteroid preparations using the Minolta Chroma Meter

CATHERINE QUEILLE-ROUSSEL, M.PONCET AND H.SCHAEFER

Centre International de Recherches Dermatologiques Galderma (CIRD Galderma), Sophia Antipolis, 06565 Valbonne Cedex, France
Centre de Pharmacologie Appliquée à la Dermatologie (CPCAD), Hopital Pasteur, 06000 Nice, France

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Summary

The Minolta tri-stimulus colorimeter CR-200 was used to quantify the blanching effect of topical corticosteroids in a non-occluded vasoconstriction test. To investigate the influence of time on variations in colorimetric parameters, an initial series of measurements was performed on Day 1 on six predetermined sites on the ventral surface of the forearm of six healthy volunteers every 2 h over a 12-h period. The colorimetric values were shown to be site related but hourly variations occurred with similar profiles for all the sites. On Day 2, four topical corticosteroid creams, representative of their potency groups, as well as a base were applied in a randomized double-blind manner on five of the predetermined sites. Visual gradings and colorimetric measurements were carried out every 2 h over the following 12-h diurnal period and were continued on Day 3. The colorimetric parameters L* (luminance) and a* (colour hue ranging from green(−) to red (+)) gave a rank order correlated to corticosteroid potency that showed superior discrimination compared to simple visual grading. In this study, L* was a more discriminative parameter than a*. The Minolta Chroma Meter CR-200 appears to be a simple and accurate device for objectively measuring the blanching effects of topical corticosteroids.

The vasoconstrictor assay[1] (human skin-blanching assay) is a well-established method for ranking topical steroids. Cornell and Stoughton[2,3] gave a rank for U.S. formulations with seven orders of potency and in Europe, the current classification for topical corticosteroid has four ranks,[4–5] with Group I being the most potent. In this assay the visual assessment of blanching generally gives good results, but under some circumstances is not a very satisfactory method, e.g. non-occluded tests for weak or moderately potent formulaions. Many studies using quantitative instrumental methods have attempted to improve the objectivity of this test. Some have completely failed[6–8] and those that succeeded were too cumbersome and time-consuming for general use.[9–11] The only study using a colorimetric system[12] was not convincing and this method was abandoned.

Recently, a hand-held tri-stimulus colorimeter was developed by Minolta which was initially employed for evaluation of erythema and tanning[13–15] and for the quantification of sodium lauryl sulphate irritant dermatitis.[16] In the present study, a Minolta Chroma Meter model CR-200 was used to measure the blanching effect of corticosteroids on normal skin. Four well-known commercially available creams, representative of the four potency groups, and a cream base were tested. The main purpose of this study was to determine whether the same order of potency could be obtained from quantification of skin blanching using colorimetric values. In order to obtain general blanching profiles, multiple readings were performed every 2 h during two consecutive diurnal periods.

Methods

Subjects

Six healthy male Caucasian volunteers aged 21–30 years were included in the study. Subjects undergoing either local or systemic treatment were not admitted. Written informed consent was obtained from each volunteer and the protocol was approved by the local Ethical Committee. The study was performed at the Centre de Pharmacologie Clinique Appliquée à la Dermatologie (C.P.C.A.D.) in Nice.

Correspondence: Dr C.Queille-Roussel, CIRD Galderma, Sophia Antipolis, 06565 Valbonne Cedex, France.

Products

Four commercial steroid creams were evaluated.

(a) Group I (very high potency): Dermovate cream (Glaxo), clobetasol propionate 0·05% w/w.

(b) Group II (high potency): Betnovate cream (Glaxo), betamethasone as valerate 0·1% w/w.

(c) Group III (moderate potency): Tridesonit cream (Dome/Hollster-Stiers, Miles), desonide 0·05% w/w.

(d) Group IV (mild potency): Hydrocortisone Astier à 1% cream (Astier), hydrocortisone 1·0% w/w.

A cream base without corticosteroid (Glaxo) was used as a control.

Colorimeter

The Minolta Chroma Meter CR-200 is a portable instrument with a flexible hand-held probe which can be moved very easily from one site to another (measuring head weight: 651 g). The measured area is 8-mm in diameter. This reflected-light colorimeter offers five different colour systems for measurement. The L* a* b* system recommended by the CIE (Commission Internationale de l'Eclairage)[17] seems to be particularly convenient for skin-colour assessment. The colour is expressed in a three-dimensional space. The L* value (luminance) gives the relative brightness ranging from total black ($L^*=0$) to total white ($L^*=100$). The a* value represents the balance between red (positive values) and green (negative values). The b* value represents the balance between yellow (positive values) and blue (negative values). Theoretically, a skin-blanching effect should increase the L* value and decrease the a* value. Before use, the calibration channel 00 of the colorimeter was brought to standard white-plate level (Calibration plate CR-A43). Calibration was performed each time the instrument was used.

Experimental design

The test took place on three consecutive days. On Day 1, spontaneous diurnal variations were studied by measuring site colour every 2 h from 08.00 to 20.00. Six circular sites (2·2-cm diameter) corresponding to the external edges of the probe were outlined with a dermatological dark pencil on the anterior aspect of the forearms. The distal part of the wrist, the veins and skin covered with hairs were carefully avoided. The head of the apparatus was placed in contact with the site to be measured with minimal pressure on the skin. The measurements were carried out by the same investigator throughout the study. At each time point, six measurements were made at each site. The position was standardized during the study and the forearms were placed in a horizontal position on a table and kept motionless for at least 2 min before the measurements.

On Day 2, a non-occluded vasoconstriction test was performed and blanching observations were further continued on Day 3. At 08.00 (t_0), on Day 2, immediately after colorimetric evaluation, 10 μl of each cream was pipetted on to the skin site at the centre of the circle, in a double-blind randomized manner and gently massaged for 30 s. One of the sites was left as an untreated control. A non-occlusive dressing (a rubber ring covered with a gauze and held in place by Scanpor Norgeplaster) prevented any loss of cream. Two hours later (t_{0+2h}) the protection was removed and the excess product was carefully wiped away using a paper tissue. A visual assessment of blanching was made in a double-blind manner by a trained observer, according to the following table of scores: 0, no changes in skin colour; 1, slight (barely visible) blanching; 2, obvious blanching; 3, intense blanching; 4, maximal blanching. Intermediate scores (of half of a unit) were used when necessary. The colorimetric readings were then made. Assessments were performed on Day 2 and Day 3 in the same way and at the same times as Day 1 by the same investigator. Room temperature was kept constant at 21–22°C and the relative humidity at 35%.

Statistical methods

Analysis of variance was used to evaluate the variations due to sites, measurements, hours and days. The products were compared using the Student–Newman–Keuls test which adjusts for multiple comparisons. The Fischer–Snedecor F statistic resulting from the between products comparisons, which reflects overall differences between products, was used as a criterion for comparing discriminative abilities of each variable at each time point. When there is no difference between products, the F statistic is close to unity.

Results

Visual assessments

With each preparation, the results for all volunteers were expressed as mean scores (six measurements × six subjects) for each time point (Fig. 1). As expected, 0·05% clobetasol propionate (Group I) induced the strongest blanching effect that differed significantly from the other

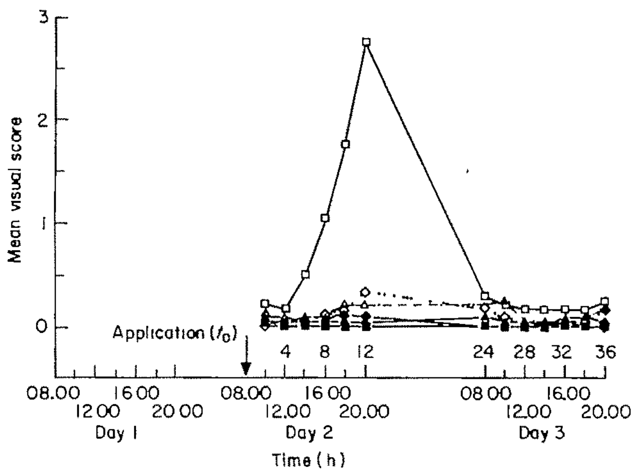

Figure 1. Visual score means $(n=6)$ as a function of time after unoccluded application (time t_0). □——□, 0·05% clobetasol propionate cream; ◇---◇, 0·1% betamethasone valerate cream; △----△, 0·05% desonide cream; ▲——▲, 1% hydrocortisone cream; ◆——◆, base cream; ■——■, untreated site.

creams. Betamethasone valerate (0·1%) (Group II) and 0·05% desonide (Group III) both expressed weaker blanching activities with no clear-cut drug-related difference. Hydrocortisone (1%) (Group IV) induced little or no blanching. After 28 h, 0·05% clobetasol propionate was the only cream still expressing an obvious blanching activity. Erythema was observed on clobetasol propionate-treated sites of two subjects 28–36 h after application.

Colorimetric assessments

Mean values of L*, a*, b* were calculated for each site before (D1) and after (D2 and D3) cream applications. Figure 2 shows mean values as a function of time.

Analysis of results obtained from untreated sites (all the sites at D1 plus untreated control sites at D2 and D3). Results from pretreatment period and untreated sites show differences in baseline values between the various sites of the forearms. Variations during the day show similar profiles for all the untreated sites. Untreated-site L* and b* values obtained during the three consecutive diurnal periods show significant reproducible profiles with a highest value at 08.00 and a lowest at 16.00. The significant decrease of a* from D1 to D3 for all volunteers may be due to the influence of treated sites or to uncontrolled non-specific events.

The Day 1 data were also used to evaluate the reproducibility between successive measurements and the random (non-systematic) variations between zones. Analyses of variance performed at each hour allowed the estimation of the standard deviation between successive measurements (0·21 for a*, 0·20 for L*, 0·12 for b*) and among zones (0·47 for a*, 0·61 for L*, 0·56 for b*). When adjusting colorimetric values by a baseline value

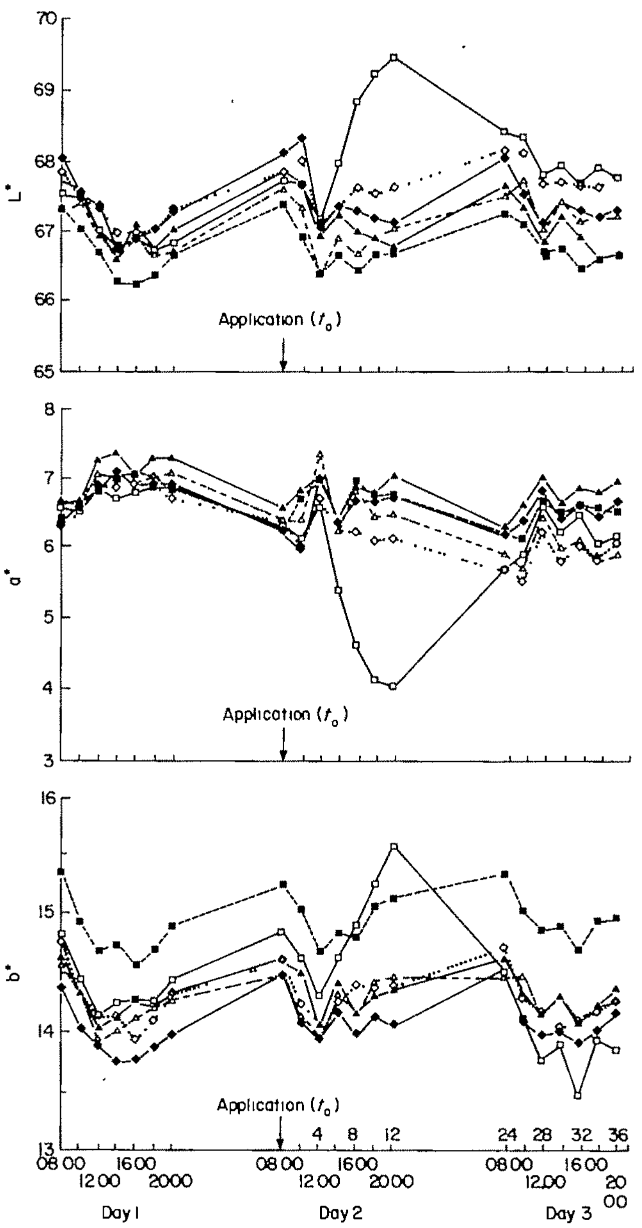

Figure 2. Absolute colorimetric values of (a) L*, (b) a* and (c) b* as a function of time. Day 1, before treatment; Day 2, unoccluded application at time t_0; Day 3, no application. □——□, 0·05% clobetasol propionate cream; ◇---◇, 0·1% betamethasone valerate cream; △----△, 0·05% desonide cream; ▲——▲, 1% hydrocortisone cream; ◆——◆, base cream; ■----■, untreated site.

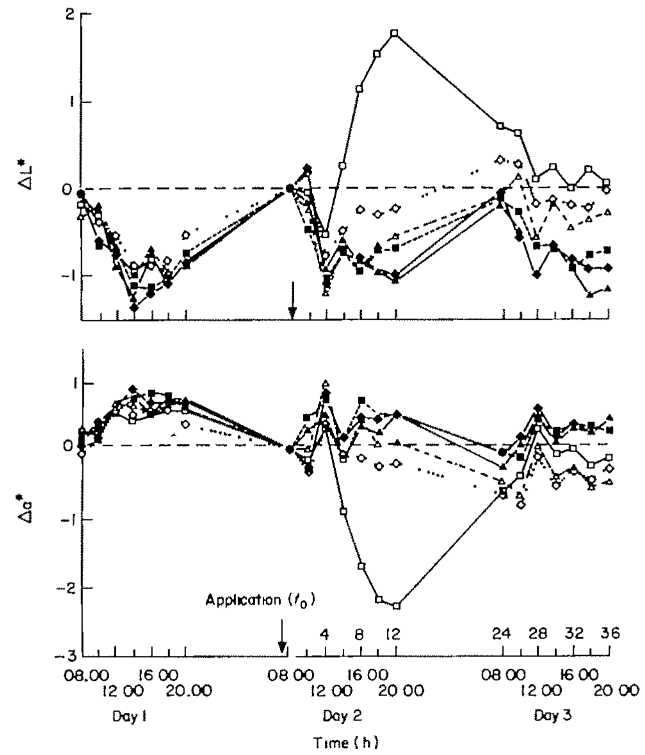

Figure 3. Colorimetric values adjusted to their own baseline value measured at time t_0 (Day 2, 08.00) as a function of time: (a) L*, (b) a*. □——□, 0·05% clobetasol propionate cream; ◇---◇, 0·1% betamethasone valerate cream; △----△, 0·05% desonide cream; ▲——▲, 1% hydrocortisone cream; ◆——◆, base cream; ■----■, untreated site.

(e.g. value at t_0), variations due to zones were reduced by 20% for a*, 25% for L* and 60% for b*.

Analysis of the vasoconstrictor assay

Comparison of products. In order to compare the colour variations induced by the four corticosteroid creams and the base, colorimetric values had to be adjusted to their own baseline value measured at time t_0 (Fig. 3). Adjusted values are reported as ΔL*, Δa* and Δb*. The calculation of area under the curve is statistically debatable because of the lack of nocturnal values. This confers unequal importance to first and last measurements each day. Therefore, in order to quantify all the blanching phenomena induced by drugs within the first 36 h following non-occluded application, the algebraic sums of mean adjusted values were selected as end results (Σ ΔL*, Σ Δa* and Σ Δb*). Rank orders obtained from these end values for both L* (Fig. 4b) and a* (Fig. 4c) parameters were correlated to corticosteroid potency groups; b* gave unrelated results (Fig. 4d). The relevance of b* variations occurring during corticosteroid-induced vasoconstriction remains obscure especially on the 0·05% clobetasol propionate-treated site.

Statistical comparisons of products performed on ΔL*, Δa* and Δb* indicate that overall, ΔL* and Δa* showed discrimination between the creams of potencies I, II, III and IV, although 1% hydrocortisone is not differentiated from the base cream and untreated site (Table 1).

With a limited number of subjects the power to detect differences is low. This group was necessarily limited to allow measurements to be taken within 50 min for each time point. Increasing volunteer numbers should allow statistically significant differences between 0·05% desonide and other groups to be obtained.

Comparison of parameters and time points. A comparison of parameters and time points in terms of discriminative power was made using the *F*-statistics from the analysis of variance (Table 2). A large *F*-statistic indicates that the differences among products are large. An *F*-statistic close to unity would indicate that differences among products are random variations, consistent with experimental variations. It is clear from Table 2 that *F*-statistics are greater for ΔL* than for Δa*, and also that $t_{(0+12)}$ h is the optimal time point from the design in terms of its ability to account for differences among these products.

Results at 12 h. Further analysis of the $t_{(0+12\,h)}$ ΔL* data (Table 3) shows that not only are this time and parameter the optimal set of conditions, but also that for four out of the six subjects, the means of ΔL* values can be ranked in exactly the same order as the clinical potencies of the creams. For the fourth and the sixth subjects, there is only an inversion of the two weakest groups.

Discussion

The aim of this study was to demonstrate that the colorimeter could measure a corticosteroid-induced blanching effect and reproduce a rank order of the potency of the test formulations. Visual scoring was used for comparison (one observer only would have been questionable in a screening test). Although the majority of topical corticosteroid formulations present optimum vasoconstriction at around 16 h when the test is unocclusive,[18] to demonstrate the sensitivity of the device it was decided to detect the onset of blanching rather than its maximum. A classification of topical corticosteroids was established in 1977[17] following a

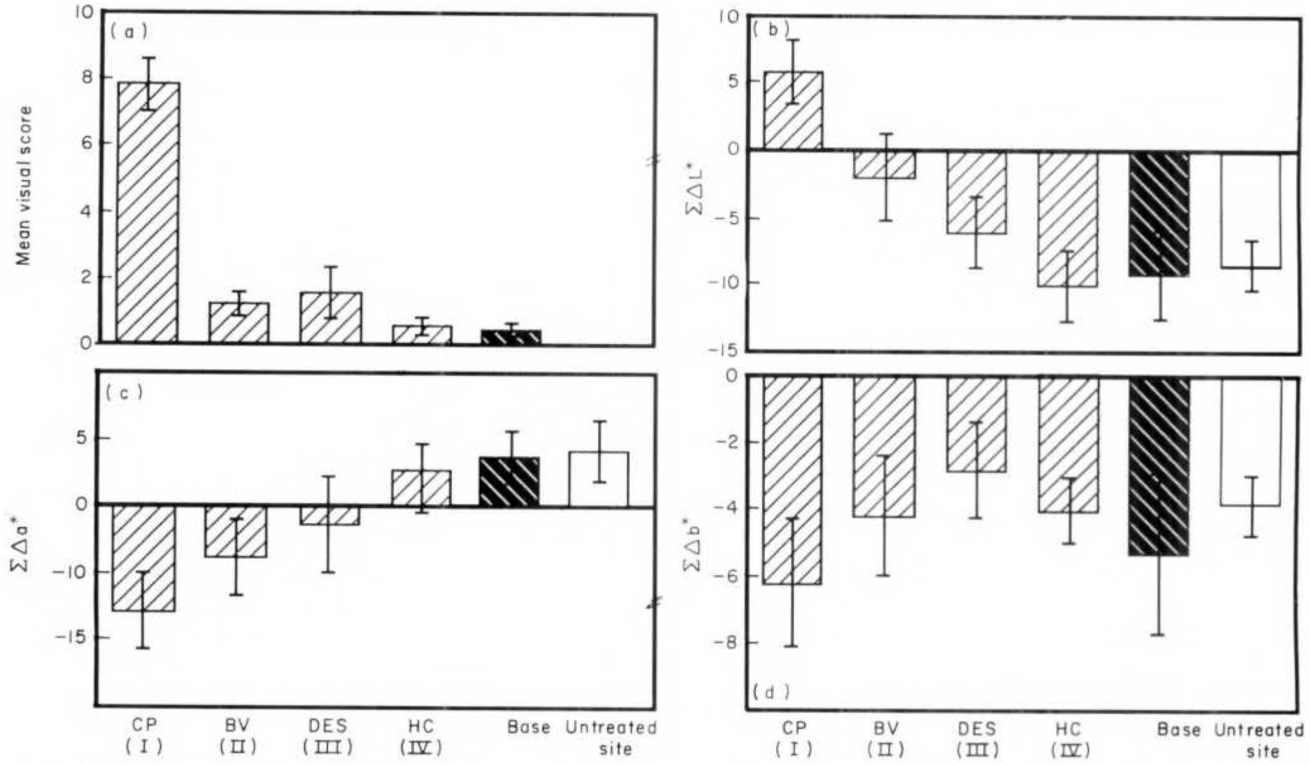

Figure 4. (a) Algebraic sums (+/− standard error) of mean visual scores obtained from time t_0 (Day 2, 08.00) to t_{36} (Day 3, 20.00). Algebraic sums of mean adjusted values (+/− standard error) of (b) $\Sigma\Delta L^*$, (c) $\Sigma\Delta a^*$ and (d) $\Sigma\Delta b^*$, measured from time t_0 (Day 2, 08.00) to time t_{36} (Day 3, 08.00). CP, 0·05% clobetasol propionate cream; BV, 0·1% betamethasone valerate cream; DES, 0·05% desonide cream; HC, 1·0% hydrocortisone cream.

Table 1. Statistical comparisons of products performed on ΔL^*, Δa^* and Δb^* (Student–Newman–Keuls test, $P=0{\cdot}05$).

	ΔL^*	F	Δa^*	F	Δb^*	F
$t_{0+12\,h}$	CP > BV > HC base CP > DES untr.	45	CP < others	39	CP > others	10
D2	CP > others	22	CP < others BV < untr.	19	CP > base	3
D3	CP > HC base untr. BV > HC base	7	ND	3	CP < others	7
D2 + D3	CP > BV > DES HC base untr.	21	CP < BV < HC DES base untr	14	ND	2

F, Fischer statistic; CP, 0·05% clobetasol propionate cream; BV, 0·1% betamethasone valerate cream; DES, 0·05% desonide cream; HC, 1% hydrocortisone cream; base, base cream; untr., untreated site; ND, no significant differences.

Table 2. *F* values at each time point for each colorimetric parameter

$t(t_{0+h})$	2	4	6	8	10	12	24	26	28	30	32	34	36
L*	3	2	6	20	29	45	3	6	6	3	3	8	6
a*	3	2	5	19	26	39	2	3	2	2	2	4	5
b*	1	0	1	3	6	10	3	3	5	3	9	7	8

Table 3. $\Sigma\ \Delta L^*$ values (rank order) at $t_{0+12\ h}$

Subject	CP	BV	DES	HC
S1	0·54 (1)	−1·01 (3)	−0·63 (2)	−1·06 (4)
S2	2·14 (1)	−0·26 (2)	−0·45 (3)	−1·03 (4)
S3	2·08 (1)	0·01 (2)	−1 15 (3)	−1·72 (4)
S4	2·56 (1)	0·96 (2)	0·29 (3)	0·12 (4)
S5	2·06 (1)	0·30 (2)	−0·08 (3)	−0 47 (4)
S6	1·05 (1)	−1 42 (3)	−1·32 (2)	−2·20 (4)
Sum	10·43 (1)	−1·42 (2)	−3·34 (3)	−6 36 (4)
Mean	1·74	−0·24	−0 56	−1·06
SEM	0 31	0·35	0·25	0·34

CP, 0 05% clobetasol propionate cream; BV, 0·1% betamethasone valerate cream; DES, 0·05% desonide cream; HC, 1% hydrocortisone cream.

trial of their comparative clinical effects in the treatment of psoriasis. This classification was compared with results obtained with the same drugs during vasoconstrictor tests.[19] The statistical analysis of these results as observed at 12 h enabled the products to be grouped into four classes of activity identical to those obtained earlier in clinical assays, and the same order as in the European classification.[4–5] In our study, 12-h measurements were performed on Day 2. Analysis at that time already gave the expected rank but multiple readings were obtained as in other studies[20–22] and these showed significant differences between 0·1% betamethasone valerate and 0·05% desonide ($\Sigma\ \Delta L^*$) and between 0·05% desonide and 1% hydrocortisone ($\Sigma\ \Delta a^*$).

Skin colour variation could be measured, whatever their nature or cause, by using reflectance spectrophotometry which succeeded in quantifying the degree of vasoconstriction induced on normal skin by topical steroids. Leveque *et al.*[11] designed and built a device which measured the skin reflectance value without any skin contact and in the blanching assay, they obtained a good correlation with clinical potency. However, this method is not suited for routine clinical use. Recently, Bisgaard *et al.*[23] re-evaluated the possibility of using laser-Doppler velocimetry by introducing the prostaglandin-dependent reactive hyperaemia reaction in the test procedure. However, this method could be performed only on the forearms and is not suitable for following up the phenomena over time.

In our opinion, the present method has a number of advantages. The device is easy to handle and relatively cheap, the time for each measurement is short and the space occupied by the measuring head is minimal causing no modification of neighbouring sites during measurement. The possible modification of skin colour due to variations in pressure on the skin could be restricted by using the same trained investigator for all measurements and by repeating the measurements each time. Statistical analysis of these results showed that between-measurement variations for each site were small compared to between-site variations and the number of measurements could be reduced in further studies. Standardization of the position of the forearm for a few minutes before and during the measurements was essential. Absolute values exhibited site- and time-related variations and must therefore be adjusted to baseline values. Two kinds of adjustment could be made here, either to the corresponding values measured for the same site at the same hour on Day 1 (unreported results) or more simply to the immediately preceding application value (at 08.00 on Day 2: t_0). As there was no additional discrimination between the product using the first method, the second and simpler one was selected.

For extensive use of the colorimeter, the calibration should be systematically checked on commencing each series of measurements but especially if it has been switched off during the experiment.

In conclusion, this colorimetric method allows an objective evaluation of cutaneous blanching provided that simple guidelines are maintained. We chose those conditions that posed problems to investigators relying on visual assessment alone and with these the colorimetric parameters L* and a* gave a rank order identical to the potency ranking of corticosteroids. Analysis of ΔL^* and Δa^* values obtained from measurements performed 12 h after a 2-h non-occluded application was found to be sufficient to give an objective and precise evaluation of topical corticosteroid potency.

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Destruction of tumour parenchyma in basal cell carcinoma by tumour-associated neutral proteases: a histochemical study

BETTINA SCHLAGENHAUFF, C.KLESSEN, SONJA TEICHMANN-DÖRR,*
H.BREUNINGER* AND G.RASSNER*
*Departments of Anatomy and *Dermatology, University of Tübingen, Germany*

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Summary Proteolytic activity was demonstrated histochemically in frozen sections of basal cell carcinomas (BCCs). After incubation of tissue sections in 0·1 M phosphate buffer with 0·25 M NaCl the tumour epithelium was almost completely destroyed. The basal and squamous cell layers of the epidermis disintegrated to varying degrees, particularly where they were directly in contact with tumour epithelium. Serine and metalloprotease inhibitors diminished this tissue destruction. Iodoacetate enhanced tumour destruction, urea and potassium thiocyanate even more so. The high proteolytic activity of BCC demonstrated in this study may be an important factor in the proliferative, invasive and destructive behaviour of this tumour.

Enhanced proteolytic activity has often been observed in malignant tumours, indicating that proteases are implicated in the proliferation, invasion and metastasis of these tumours. It is generally assumed that proteases secreted either by the tumour cells themselves or by host cells degrade the constituent elements of the extracellular matrix and thus promote tumour spread.[1] It appears probable that a broad spectrum of specific proteases is involved in this process and particular importance is attributed to collagenases, plasminogen activators, and cathepsin B. Various studies have also indicated that proteases change the surface structure of cells, thus weakening cell adhesion.[2] Cell division has been found to be stimulated by proteases.[3] In addition, tumour induction and promotion can be affected by proteases.[4,5]

Basal cell carcinoma (BCC) is a common skin tumour characterized by local infiltrative and destructive growth, but with very little metastatic potential. Collagenolytic activity has been shown to be greater in BCC than in normal skin.[6,7] The same studies have shown that tumour-related collagenase was localized to the connective tissue around the tumour and was secreted by fibroblasts. Goslen *et al.*[8] discovered cytokines produced by BCC cells capable of stimulating the secretion of collagenase in the fibroblasts.

The aim of this study was to investigate proteolytic activity in BCC tissue. A simple histochemical technique with salt-containing phosphate buffer was used to detect proteases and demonstrate their high potential for tissue destruction. Specific protease inhibitors were used for more precise characterization of the proteolytic activity.

Methods

Twenty-five basal cell carcinomas (BCCs) from the head were studied. Normal skin removed during plastic surgery was used as a control. The skin specimens were cut into small pieces, embedded in Tissue Tek, and frozen in isopentane cooled with liquid nitrogen. Cryostat sections 10 μm thick were cut and mounted on microscope slides. Following a maximum drying period of 20 min, 2 ml of buffer solution were dropped onto each slide and the slides incubated in a wet chamber. After incubation with phosphate buffer containing NaCl, there was marked destruction of the tumour epithelium and the basal layers of the epidermis to varying degrees. This was interpreted as evidence of proteolytic activity.

Optimum conditions for incubation were ascertained in a preliminary study of factors such as incubation period, temperature, pH, salt concentration and phosphate buffer molarity. The following conditions gave best results: incubation of cryostat sections with 0·1 M phosphate buffer at pH 7·4 with addition of 0·25 M NaCl for 150 min at 39°C (standard incubation).

Specific protease inhibitors were used to determine which of the protease classes (biochemically defined as serine, metallo-, cysteine, and aspartate proteases) included the proteases causing tissue destruction. The

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Correspondence: Professor C.Klessen, Anatomisches Institut, Östebergstr. 3, D-7400 Tübingen, Germany.

following inhibitors were used. Serine protease inhibitors: aprotinin (Bayer, Leverkusen, Germany); soy bean trypsin inhibitor (SBTI; Serva, Heidelberg, Germany); FUT-175 nafamstat mesilate (FUT; Torii, Tokyo, Japan); N-tosyl-L-lysine chloromethyl ketone (TLCK; Fluka, Buchs, Switzerland); P-tosyl-L-phenylalanine chloromethyl ketone (TPCK; Fluka); phenylmethylsulphonyl fluoride (PMSF; Sigma, Deisenhofen, Germany). Metalloprotease inhibitors: ethylene diamine tetra-acetic acid (EDTA; Serva); 1·10-phenanthroline (Serva). Cysteine and serine protease inhibitor: leupeptin (Fluka). Aspartate protease inhibitor: pepstatin (Bachem, Bubendorf, Switzerland). Cysteine protease inhibitor: iodoacetate (Merck, Darmstadt, Germany).

These inhibitors were tested in two different sets of experiments: (i) simultaneous incubation, that is, incubation of the tissue sections with inhibitor dissolved in standard incubation medium for 150 min; (ii) Pre-incubation with each inhibitor in 0·1 M phosphate buffer pH 7·4 for 30 min at 39°C followed by incubation for 150 min with NaCl phosphate buffer (standard incubation medium).

The effects on tissue destruction of urea (1 M and 3 M) and potassium thiocyanate (Merck, 0·5 M and 2 M) were also studied by means of simultaneous incubation.

After incubation, the medium was carefully poured off and the sections fixed for 30 min in a 4% formol-calcium mixture. The tissue sections were then washed for 5 min in tap-water, rinsed in water, and stained for 2 min with toluidine blue solution.[9] They were rinsed in water, air-dried, dehydrated in xylene, and mounted with DePeX. The extent of tissue destruction was assessed by an observer with no knowledge about the experimental conditions.

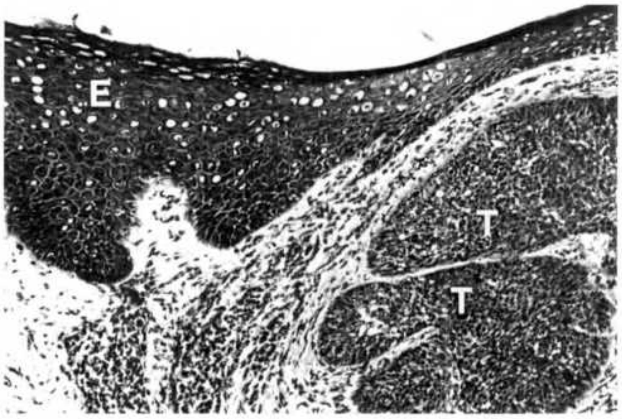

Figure 1. Solid BCC showing T, tumour nodule; E, epidermis and inflammatory cell infiltrate in the dermis (toluidine blue, × 40).

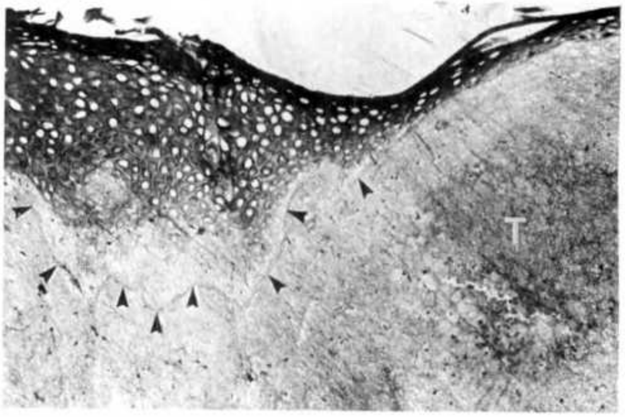

Figure 2. Solid BCC after incubation with 0·25 M NaCl phosphate buffer: the basal cell layer of the epidermis and the cellular infiltrate have disintegrated. Arrows indicate the dermo-epidermal junction. T, tumour epithelium (toluidine blue, × 40).

Results

Incubation with 0·25 M NaCl phosphate buffer pH 7·4

Almost complete destruction of tumour tissue appeared after incubation of the sections with 0·1 M phosphate buffer pH 7·4 containing 0·25 M NaCl. The cell nuclei showed dust-like disintegration, but were occasionally also hyperchromatic and pyknotic (Figs 1 and 2). Pyknotic cell nuclei often had a pronounced surrounding halo of lysis (Fig. 3). In the epidermis, the basal cell layer and the inferior squamous cell layers were primarily affected, particularly near the rete ridges and at points of contact with the tumour epithelium. The keratinocytes were barely stained in these areas and appeared almost structureless (Fig. 2). In some cases, clefting at the dermo-epidermal junction zone was observed. The

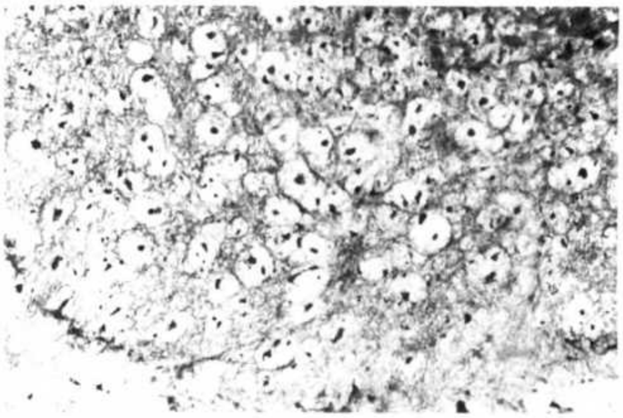

Figure 3. Solid BCC after incubation with 0·25 M NaCl phosphate buffer: 'halos' around nuclear residues inside the destroyed tumour nodule (toluidine blue, × 100).

Table 1. Dependency of tissue destruction on pH. Semiquantitative evaluation of results of incubation

pH	Tumour cells	Epidermis
5·0	—	—
6·0	+ +	+
7·0	+ + +	+ +
7·4	+ + +	+ + +
8·3	+ + +	+ + +

+ + +, Maximum destruction: cells dissolved, dust-like disintegration of the nuclei, scattered nuclear pyknoses with halos; + +, pronounced destruction of cells: cell nuclei partly disintegrated, partly well preserved, but loosely structured; +, only mild signs of destruction: cytoplasm and cell nuclei show slight structural loosening; —, no signs of lysis.

epidermis of normal skin showed only discrete lytic changes in the area of the basal cell layer. Also affected were the inflammatory cell infiltrate and the outer layers of the epithelial hair root sheaths. Lytic changes were less marked following incubation of the tissue sections with NaCl-free phosphate buffer.

Results of the preliminary tests

Tissue destruction reached the greatest extent after an incubation period of 150 min. An increase of temperature from 30 to 39°C resulted in increased destruction. Tissue destruction increased up to a pH of 7·4 (Table 1). Tissue destruction increased as NaCl concentration rose above 0·1 M up to a maximum of 0·25–0·5 M, but decreased at higher concentrations (Table 2).

Table 2. Dependency of tissue destruction on salt concentration. Semiquantitative evaluation of results of incubation

NaCl (M)	Tumour cells	Epidermis
0·00	+	+
0·10	+ +	+
0·25	+ + +	+ + +
0·50	+ + +	+ + +
1·00	+ +	+(+)
2·00	+(+)	+
3·00	+(+)	(+)

See Table 1 for key.

Inhibition tests

Tissue destruction was inhibited when specific protease inhibitors were used (Table 3).

Simultaneous incubation. The serine protease inhibitors PMSF, FUT and TLCK effectively inhibited tissue destruction (Fig. 4). Tumour islands were clearly delineated, and the structure of the epidermal layers was well preserved, even though there were discrete signs of disintegration in the tumour cells and the basal cell layer of the epidermis. SBTI and aprotinin were less inhibitory. Tissue destruction was clearly reduced by the metalloprotease inhibitor EDTA and even more so by phenanthroline. Although leupeptin displayed an inhibitory effect on epidermal destruction, it did not significantly inhibit disintegration of the tumour. No inhibition was observable with pepstatin and TPCK. The cysteine protease inhibitor iodoacetate led to increased tissue lysis, espe-

Table 3. Inhibition tests: (a) Simultaneous incubation with inhibitors in NaCl-phosphate buffer. (b) Pre-incubation with inhibitors in NaCl-free phosphate buffer and subsequent incubation with NaCl-phosphate buffer

		(a)		(b)	
Inhibitors	Concentration	Tumour	Epidermis	Tumour	Epidermis
Aprotinin	0·28 mg/ml	1	1	2	2
SBTI	0·5 mg/ml	1	1	2	2
PMSF	2×10^{-3} M	2	2	2–3	2–3
FUT	2×10^{-4} M	1–2	1–2	2	2
TLCK	10^{-2} M	1–2	1–2	2–3	2–3
TPCK	10^{-3} M	0	0	2	2
EDTA	10^{-2} M	1–2	1–2	1	1
Phenanthroline	10^{-2} M	3	3	3	3
Leupeptin	10^{-3} M	0	1	0	1
Pepstatin	10^{-3} M	0	0	0	0
Iodoacetate	10^{-3} M	0*	0*	0*	0*

0, no inhibition, 1, slight inhibition; 2, good inhibition; 3, nearly complete inhibition.
* Enhanced destruction.

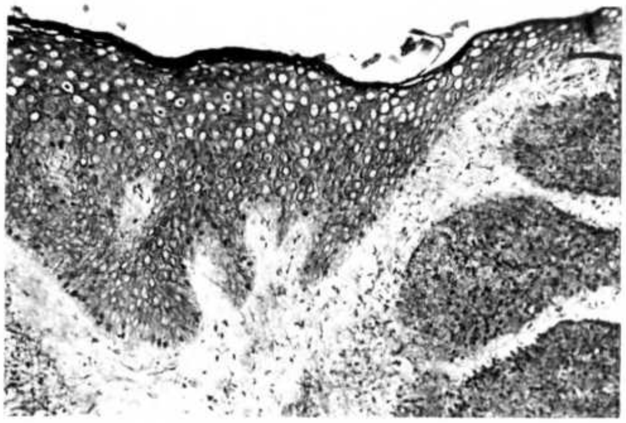

Figure 4. Solid BCC after simultaneous incubation with 0·25 M NaCl phosphate buffer+PMSF $(2\times10^{-3}$ M). The inhibition of tissue destruction is clearly visible (toluidine blue, ×40).

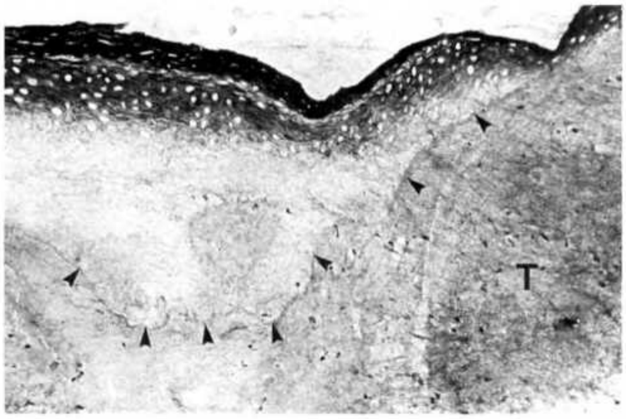

Figure 5. Solid BCC after simultaneous incubation with 0·25 M NaCl phosphate buffer+iodoacetate $(10^{-3}$ M): tissue destruction is markedly stronger than in Figure 2, especially in the epidermis. Arrows indicate the dermo-epidermal junction (toluidine blue, ×40).

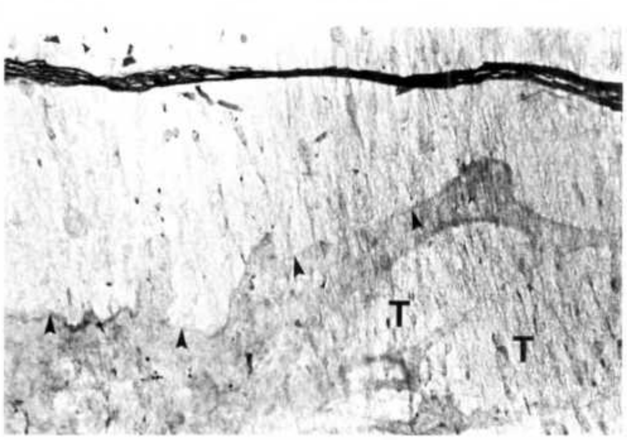

Figure 6. Solid BCC after incubation with 3 M urea in NaCl phosphate buffer: the tumour epithelium and epidermis apart from the stratum corneum have completely disintegrated (toluidine blue, ×40).

cially in the epidermis (Fig. 5). Lysis increased markedly using urea (Fig. 6) and potassium thiocyanate: the tumour epithelium and the epidermis degenerated completely apart from the stratum corneum.

Pre-incubation. The effect of most inhibitors was markedly stronger following pre-incubation than in the simultaneous incubation technique. TPCK resulted in marked inhibition in the pre-incubation experiment, but none in simultaneous incubation. No differences between simultaneous and pre-incubation were observed using phenanthroline, iodoacetate, leupeptin and pepstatin. EDTA was the only inhibitor which was more effective when added directly to the incubation medium.

Discussion

Incubation with 0·25 M NaCl phosphate buffer resulted in an almost complete disintegration of BCC tissue; this was inhibited by serine and metalloprotease inhibitors.

This tissue destruction is presumably attributable to the effect of proteases activated by incubation with salt-containing buffer solution. Proteolytic activity was found in psoriatic skin when a similar histochemical technique was used.[10] As early as 1945, Beloff and Peters[11] showed that a salt-containing buffer solution (5% KCl) must be used for the extraction of a certain skin protease. Buffer solutions containing salt have been used in a large number of studies to extract proteases from tissue homogenates.[12,13] The assumption was that these solutions separate proteases from cell membranes, ground substance and other structures or liberates them from inactive complexes.[14] Two mast-cell proteases were investigated and high salt concentrations were found to be necessary for the extraction of one of the proteases due to its firm bond with heparin, while the other was extractable without a high salt concentration.[15]

Although the tumour degradation demonstrated in this study should be related to the activation of tissue proteases, no conclusion can be drawn about the *in-vivo* release of proteases because histological sectioning and freeze-thawing disrupts cells and their organelles causing leakage of intracellular constituents.

Tissue degradation in the present study was almost entirely absent at concentrations above 1 M NaCl (Table 2). A possible explanation for this could be that neutral salts at low concentrations increase the solubility of many proteins ('salt-in' effect). However, protein solubility decreases at high salt concentrations, as proteins precipitate at a certain degree of ionic strength ('salt-out' effect). The decrease in tissue destruction observed at higher salt concentrations may therefore have been due to the 'salt-out' effect, a process in which protease activity is weakened while substrates (i.e. macromolecular proteins of cells and tissue) become more resistant to degradation, both of which result in decreased proteolysis.

The pH series and the inhibition experiments showed that serine and metalloproteases, which usually function best in neutral to weak alkaline pH range, are the probable source for the intense tissue destruction. Some inhibitors were more effective when pre-incubated with the NaCl-free phosphate buffer. Without this step activation and inhibition occurred concurrently. The inhibitory effect may be decreased due to a higher affinity of the activated proteases to substrate rather than inhibitor. Destruction was promoted by iodoacetate and, to a greater degree, by urea and potassium thiocyanate. Reagents which block thiol groups have been described as collagenase activators.[16] It is possible that the increase of destruction caused by iodoacetate, which alkylates thiol groups, was due to increased activation of proteases through chemical inactivation of endogenic inhibitors.

It is unclear to what extent the increase of lysis caused by urea should be seen as a consequence either of pronounced enzyme activation or general denaturation processes. The lytic effect of subsequent incubation with urea decreased markedly after pre-incubation with the protease inhibitors PMSF and TPCK, indicating protease involvement in the action of urea on tissue destruction. It is well known that urea can change the secondary and tertiary structure of proteins.[17] Under strong solutions of urea a globular protein with intramolecular disulphide bonds becomes unfolded. This leads to the exposure of peptide bonds which are normally inaccessible to proteases, thus facilitating degradation. This also applies to potassium thiocyanate, which destabilizes proteins.

Studying the effect of urea on the proteolytic activity of leucocyte extracts, Asghar and Cormane[18] showed that urea does not activate enzymes in the extracts but exposes the peptide bonds of the substrate, facilitating hydrolysis. Certain proteases are somewhat more resistant to denaturation under urea than their substrates, as shown with trypsin.[19]

Our experiments comparing BCCs and normal skin demonstrated markedly enhanced proteolytic activity in both the tumour epithelium and the adjacent epidermal areas. The basis of this increased proteolytic potential is presumably a shift in the physiological equilibrium between proteases and protease inhibitors caused either by a primary increase in the synthesis of proteases and/or corresponding protease activators, or by a primary deficiency of protease inhibitors. Wegener-Childers *et al.*[20] showed that both collagenase and a collagenase inhibitor are present in increased quantities in the peritumorous stroma of BCCs. They interpreted differences in their distribution in BCC as a localized imbalance of this equilibrium and considered a possible relation to tumour invasion. Accordingly, it has been proposed that both serine and metalloproteases play a role in tumour invasion.[21,22]

The question arises whether protease inhibitors as well as activating substances like urea may be of therapeutic value in the treatment of malignant tumours. Some studies have demonstrated an antitumour effect but others revealed no such effect or even a tumour-enhancing action of protease inhibitors.[23] One of the reasons for the failure of protease inhibitors in tumour therapy may be that although proteases are involved in tumour invasion and spread they are not clearly identified or characterized. Moreover the multispecificity of most of the protease inhibitors may be responsible for the inconsistency of the results.[23] The potential use of proteolysis-enhancing substances for tumour therapy has not yet been evaluated. Urea, for example, was used successfully for local treatment of basal and squamous cell carcinomas and high regression rates were achieved.[24] Our results with frozen sections of BCC indicate how such urea therapy may function.

In conclusion, the high potential for tissue destruction by proteases demonstrated in BCCs in our study suggests that these enzymes may be involved in the processes of tumour invasion and destructive growth. It is possible that these proteases affect cell division and proliferation, as with, for example, some serine proteases.[3]

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Pseudo T-cell lymphoma due to scabies in a patient with Hodgkin's disease

SHERNAZ WALTON, W.W.BOTTOMLEY, E.H.WYATT AND H.P.R.BURY*
Department of Dermatology, Hull Royal Infirmary, Hull
**Department of Histopathology, Castle Hill Hospital, Cottingham*

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Summary

We report a patient with scabies which clinically resembled secondary lymphomatous spread from her known Hodgkin's disease and histologically resembled a T-cell lymphoma with marked epidermotropism, a feature not previously reported in scabies.

Case report

A 78-year-old female presented with a lymph node mass in the neck in June 1989. A biopsy of the node showed the histology of nodular sclerosing Hodgkin's disease. Further investigations revealed no other site of involvement and she received radical radiotherapy to the neck, mediastinum and axillae. In November 1989 she presented with widespread infiltrated purplish nodules scattered over the trunk, limbs and face. There was no accompanying itch and she was otherwise asymptomatic. The initial clinical diagnosis was that of a cutaneous lymphomatous infiltrate secondary to Hodgkin's disease.

A skin biopsy was performed and this showed a dense cellular infiltrate in the upper dermis and to a lesser extent around the small blood vessels of the lower dermis. This consisted mainly of plasma cells, lymphocytes and histiocytes, but there were frequent large and atypical plasmacytoid cells, atypical mononuclear cells and Langerhans type giant cells. There was evidence of epidermotropism and occasional Pautrier microabscesses were seen (Fig. 1), as were occasional mitoses. Immunohistological staining, using UCH L1 (T-lymphocyte) and L26 (B-lymphocyte) showed the infiltrate to be almost completely of T-cell origin. Histologically the picture was felt to represent a T-cell lymphoma. However, the presence of a dense T-cell infiltrate in the dermis led to considerable diagnostic difficulty. The lymph node and skin sections were sent to Dr A.G.Stansfeld of the Histopathology Department, St Bartholomew's Hospital, London, who was in agreement with the diagnosis of nodular sclerosing Hodgkin's disease and would have been puzzled by the skin biopsy had it not been for a section that disclosed an acarus in the epidermis (Fig. 2).

Correspondence: Dr S.Walton, Department of Dermatology, Hull Royal Infirmary, Anlaby Road, Hull, N. Humberside HU3 2JZ, U.K.

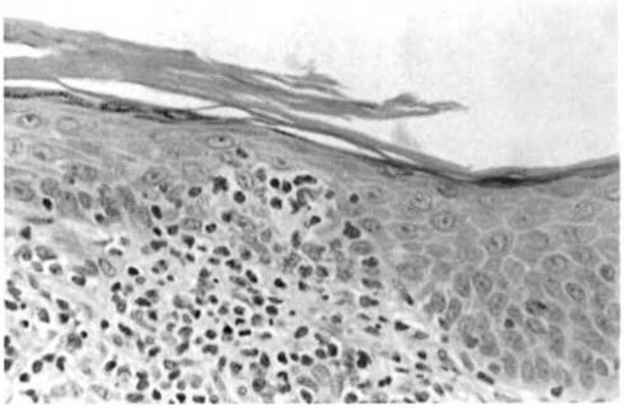

Figure 1. Dermal infiltrate with epidermotropism and Pautrier microabscesses. Haematoxylin and eosin, × 250.

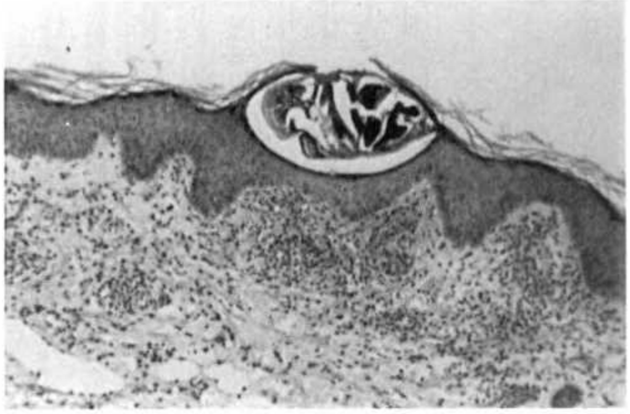

Figure 2. Acarus within the epidermis. Haematoxylin and eosin, × 100.

In view of these findings, PUVA therapy which the patient was due to start was withheld and she was treated with 1% lindane cream (Lorexane). A month later her skin lesions had almost disappeared.

Discussion

Infections and infestations, such as scabies, are not uncommon in immunocompromised individuals. The absolute lack of pruritus and the widespread distribution of the lesions, affecting such areas as the eyelids and ears with no predilection for areas usually affected by scabies were unusual features of our case. The morphology of the lesions was atypical for nodular scabies being much more succulent, infiltrated and purplish in colour than usual.

Whilst a histological picture simulating malignant lymphoma has been documented in the nodular form of scabies,[1] the infiltrate is usually located deeper in the dermis and associated with areas of free haemorrhage.[2] Our patient lacked the classical nodular clinical appearance and histologically the T-cell infiltrate showed considerable epidermotropism, atypical mononuclear cells and the formation of Pautrier microabscesses.

A histological study[3] in 39 patients with all forms of scabies showed a superficial and deep perivascular mixed inflammatory cell infiltrate comprising lymphocytes, histiocytes and numerous eosinophils. None of the cases showed epidermotropism but the lymphocytes were not stained to indicate their origin. More recent studies, including that of Reunala *et al.*[4] have demonstrated that the predominant cell in the inflammatory infiltrate is the T cell and again epidermotropism is absent. Our patient was unusual as the skin histology showed a T-cell infiltrate with epidermotropism and absence of eosinophils, features said not to favour the diagnosis of scabies. This case serves as a useful reminder that scabies can masquerade as a lymphoma and can thus be misdiagnosed.

Acknowledgments

We thank Dr A.G.Stansfeld of the Histopathology Department at St Bartholomew's Hospital, London, for his opinion on the histology and Drs M.E.Holmes and D.V.McGivern for referring this interesting patient.

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Carcinoma erysipeloides

L.R.LEVER AND P.J.A.HOLT

Department of Dermatology, University Hospital of Wales, Heath Park, Cardiff CF4 4XN, U.K.

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Summary We report an 83-year-old woman with carcinoma erysipeloides due to an occult carcinoma. Immunohistochemical study of skin biopsies showed reactivity to S-100 protein and human milk fat globule antigen indicating that the tumour originated in the breast.

Carcinoma erysipeloides, sometimes known as inflammatory carcinoma is a rare form of cutaneous metastasis with a characteristic clinical picture that can be mistaken for cellulitis. The histological picture of dermal lymphatic carcinomatosis is pathognomonic.

Case report

An 83-year-old woman was referred with a basal cell carcinoma on the bridge of the nose. This was excised and the diagnosis confirmed histologically. At operation, it was noted that she had an erythematous plaque affecting the skin of the right side of the anterior chest wall and a smaller similar plaque on the right side of the neck. The patient was uncertain of the duration of these lesions and they were asymptomatic. She was otherwise well apart from mild ischaemic heart disease controlled with nifedipine and digoxin. Examination of the plaques revealed non-tender induration with oedema and erythema reminiscent of erysipelas. The patient was afebrile and there was no axillary or cervical lymphadenopathy. A provisional diagnosis of carcinoma erysipeloides (CE) was made. General examination including palpation of both breasts was normal. Urinalysis, full blood count, urea, electrolytes and liver function tests were normal.

A biopsy was taken from each plaque and both showed similar histological features. The upper dermal lymphatics were dilated and tightly packed with an infiltrate of malignant cells showing gross pleomorphism with many atypical nuclear forms and abnormal mitotic figures. A cuff of mononuclear inflammatory cells was seen around some of the infiltrated lymphatics. Occasional individual malignant cells were also identified in the deep dermis (Figs. 1 and 2). Immunocytochemical studies were performed. There was patchy carcinoembryonic antigen (CEA) reactivity in the malignant infiltrate. The malignant cells demonstrated uniform reactivity to S-100 antigen and strong reactivity to an epithelial marker human milk fat globule antigen (HMFG II).

The histological finding of dermal lymphatic carcinomatosis supported the clinical diagnosis of CE and the immunocytochemical findings indicated that the primary tumour had arisen in the breast. A mammogram was performed which revealed segmental duct enlargement in the right breast very suggestive of a ductal carcinoma.

Treatment was commenced with tamoxifen 20 mg twice daily. There was initial improvement and after 6 weeks of therapy the plaques on the chest and neck had softened and flattened with a reduction in erythema. However, there was subsequent deterioration with enlargement and coalescence of the cutaneous plaques (Fig. 3) which were treated by radiotherapy using a direct electron field (2400 cGy in three fractions in 3 weeks). The treated area responded dramatically but she developed CE on the opposite side and a large mass in the left breast appeared with axillary lymphadenopathy. The cutaneous disease improved with further palliative radiotherapy but she developed hepatomegaly, presumably due to metastatic carcinoma, and died 15 months after presentation.

Discussion

Carcinoma erysipeloides, sometimes called inflammatory carcinoma[1] is an uncommon but distinctive form of cutaneous metastasis,[2,3] complicating approximately 1–2% of all cases of malignant breast disease.[4] The clinical features of CE were described by Lee and Tannenbaum in 1924.[5] 'Upon palpation the skin is hot, and not infrequently slight tenderness is elicited ..... becomes deep red or reddish purple and to the touch is brawny and

Correspondence: Dr L.R.Lever.

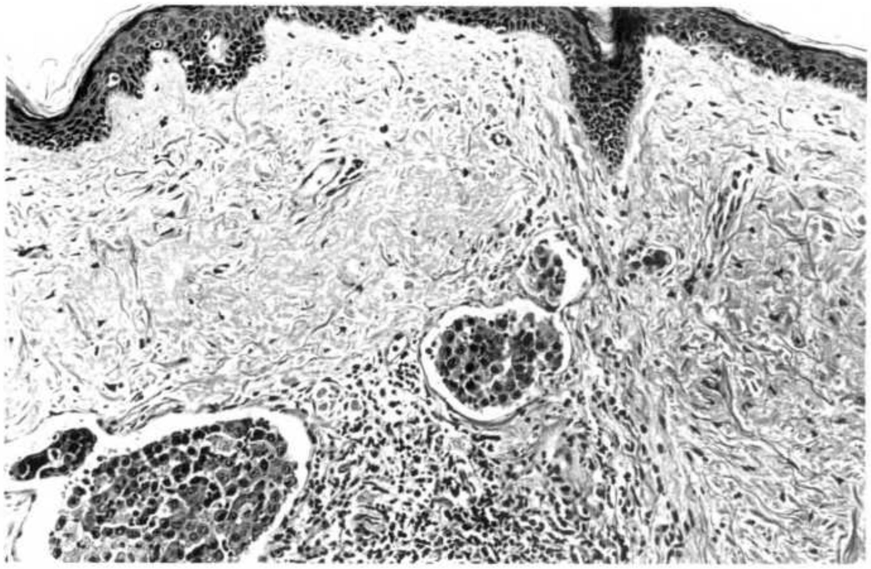

Figure 1. Dermal lymphatics dilated by tightly packed malignant cells (haematoxylin and eosin × 10).

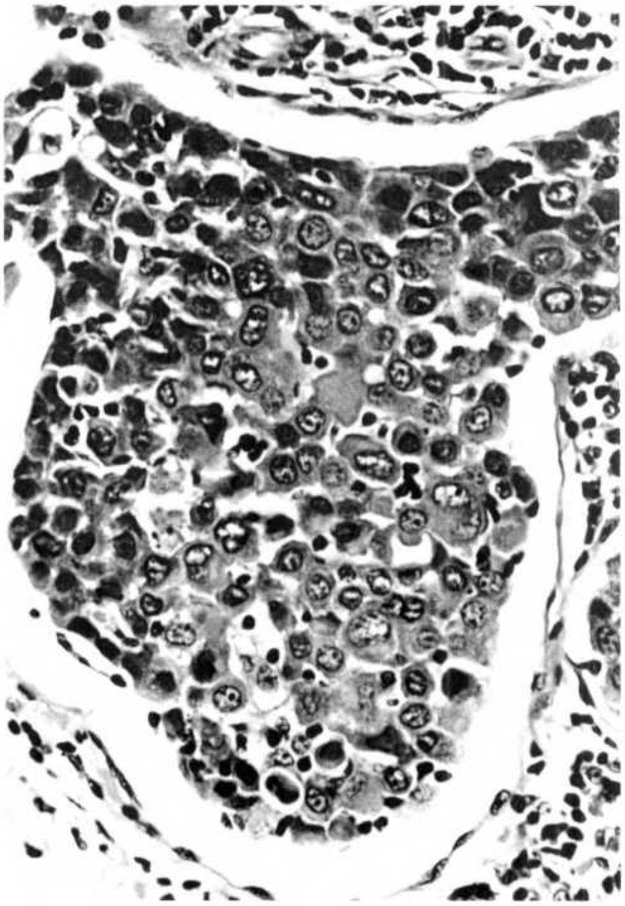

Figure 2. Pleomorphic cellular infiltrate (haematoxylin and eosin × 25).

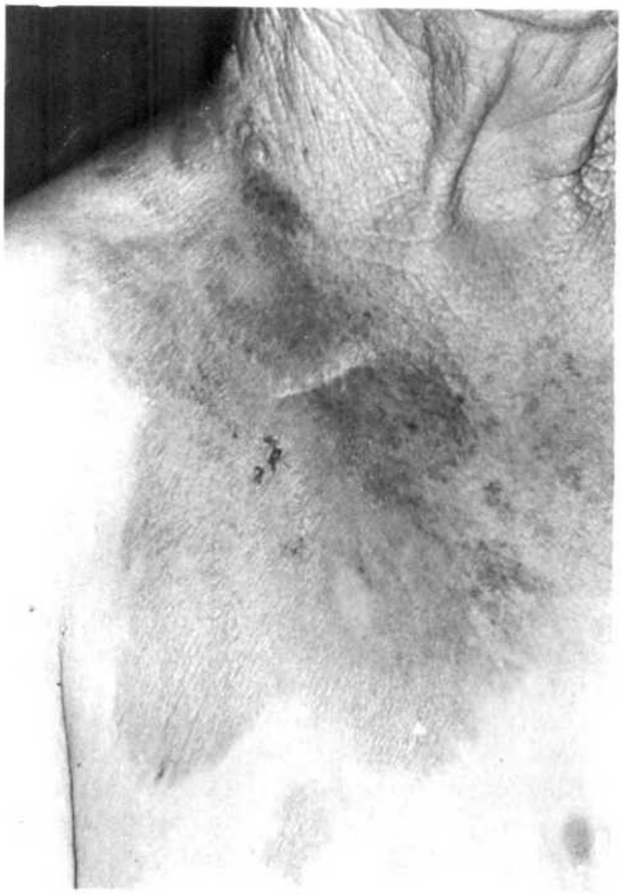

Figure 3. Erythematous plaque over right breast and neck.

infiltrated. The inflamed area presents a distinct raised periphery after the fashion of erysipelas.'

The histological features of CE are invasion of the dermal lymphatics by tumour cells accompanied by oedema and slight perivascular lymphoid infiltrate.[6] Some authors believe that breast cancer with tumour infiltration of the dermal lymphatics is accompanied by the clinical features of cutaneous oedema and erythema simulating infection in every case.[7] However, cases of 'occult inflammatory cancer' with dermal lymphatic carcinomatosis and no clinical signs of skin disease have been described.[4,8] Carcinoma erysipeloides should be distinguished from scirrhous carcinoma or *cancer en cuirasse*, in which there is non-inflammatory induration of the skin showing fibrosis and containing only a few tumour cells.[2]

The site of a cutaneous deposit and the clinical and histological features may indicate the most likely primary site of a tumour.[9] Carcinoma erysipeloides with intra-lymphatic spread of carcinoma in the skin of the chest is almost always from a poorly differentiated ductal carcinoma of the breast.[10] However, there have been reports that identical changes affecting the skin over the breast may rarely result from primary tumours at other sites including uterus, lung, ovary, stomach, tonsils and pancreas.[5,11]

We were able to demonstrate immunoreactivity of the metastic tumour to the three antibodies tested (CEA, S-100, HMFG II). Reactivity to CEA is a feature of many tumours with metastatic potential so this finding is of little value in the identification of the tissue of origin. Epithelial tissues may be identified with antibodies to keratin proteins or epithelial membrane antigen. Antibodies to HMFG II are epithelial membrane antibodies and consistently react with many carcinomas, particularly carcinomas of breast origin.[12] Immunoreactivity to S-100 antigen in a metastatic poorly differentiated tumour is suggestive of malignant melanoma[13] but S-100 protein has also been demonstrated in some tumours of epithelial tissues such as breast,[14] salivary gland, [15] and various poorly differentiated carcinomas.[16] In our case the findings of reactivity of the tumour cells to both S-100 and HMFG II is strongly suggestive that the tumour infiltrate originated from a breast carcinoma. Stroup and Pinkus[17] recently demonstrated S-100 reactivity in 11 out of 14 cases (79%) of metastatic breast cancer and reactivity to epithelial membrane antigen in 100% of cases.

The clinical progression seen in this case is typical of reported cases of CE. There is rapid enlargement of the affected area without skin ulceration. The average life expectancy of 2 years from the time of diagnosis is not improved by surgery and immediate skin recurrence is usual as a result of diffuse lymphatic invasion.[5] Improved survival after radical mastectomy has been claimed[18] but Ellis and Teitelbaum[19] reviewed the eight reported cases and found that none had tumour invasion of dermal lymphatics, the hallmark of CE. Effective palliation of CE with radiotherapy[20] or with adrenalectomy and oophorectomy[21] has been reported. There are no reports of patients with CE treated with tamoxifen. Although tamoxifen may be effective in some cases of metastatic breast cancer,[10] our patient showed only temporary improvement with this drug. It has recently been demonstrated that there is an association between inflammatory breast carcinoma and amplified *c-myc* gene which may contribute to the rapid progression of this type of tumour.[22]

Acknowledgment

We wish to thank Dr B.Jasani for performing the immunohistochemical studies.

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Sebaceous carcinoma: an unusual cause of a rapidly enlarging rhinophyma

R.J.MOTLEY, A.F.DOUGLAS-JONES* AND P.J.A.HOLT

*Departments of Dermatology and *Pathology, University Hospital of Wales, Heath Park, Cardiff, U.K.*

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Summary We describe a patient who developed sebaceous carcinoma within a long-standing rhinophyma with rapid enlargement of the lesion. We believe the development of this tumour was a random occurrence but the possibility of tumour development should be considered in any rhinophyma showing a rapid increase in size.

Most cases of true sebaceous gland carcinoma develop on the eyelids, but extra-ocular lesions may present as nodular tumours on the head and neck of elderly patients. These tumours may metastasize and should be treated with wide local excision. We describe a patient who developed this unusual tumour in a long-standing rhinophyma.

Case report

A 91-year-old man presented with an ulcerated massive rhinophyma (Fig. 1). The lesion had been present for over 20 years and had caused him little concern. Over the preceding 12 months, however, it had enlarged progressively, culminating in ulceration and a persistent serous discharge. The increased size of the lesion had produced difficulties in breathing, and prompted his referral for treatment. He had a past medical history of a benign oesphageal diverticulum and intermittent dysphagia, but was otherwise well. On examination, the rhinophyma measured 6 cm in diameter and there was ulceration distally and redundant tissue partially occluding the nares. Physical examination was otherwise unremarkable. In view of the size and vascularity of the lesion he was treated with a carbon dioxide laser. Under general anaesthesia an attempt was made to pare the lesion using a focused carbon dioxide laser beam. Progress was very slow and subsequently changed to cutting diathermy to resect the bulk of the tissue. The patient was symptomatically much improved by this procedure. The resected material was submitted for routine histological examination and we were surprised to discover the presence of a tumour within the rhinophyma tissue.

Correspondence: Dr R.J.Motley

The tumour consisted of irregular lobules of cells separated by fine fibrovascular stromal septae (Fig. 2). Many of the cells had abundant foamy cytoplasm and there was considerable variation in the size and shape of the nuclei. A number of mitotic figures were also seen. Stains for keratin, low-molecular-weight keratin (Cam 5·2), glycogen, mucin, prostatic acid phosphatase and S-100 were negative. Stains for carcinoembryonic antigen showed strong positive staining in individual and groups of tumour cells but the majority of the tumour

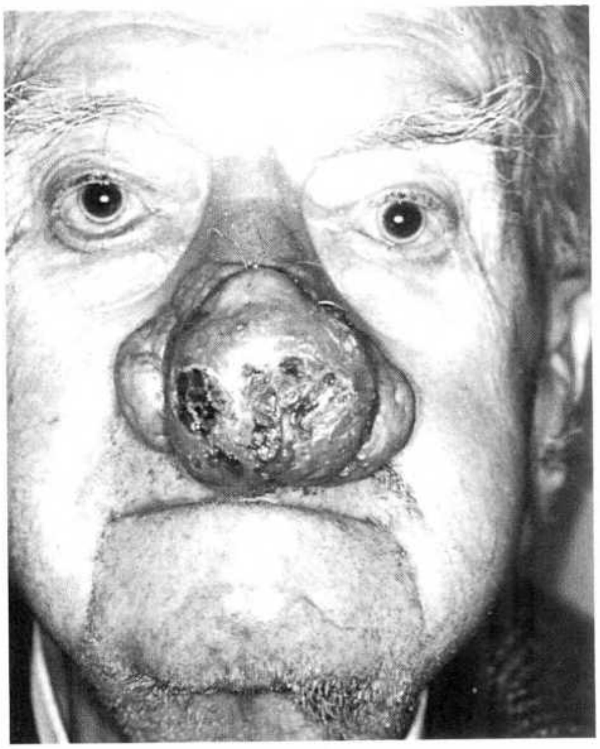

Figure 1. Preoperative frontal view of the ulcerated rhinophyma.

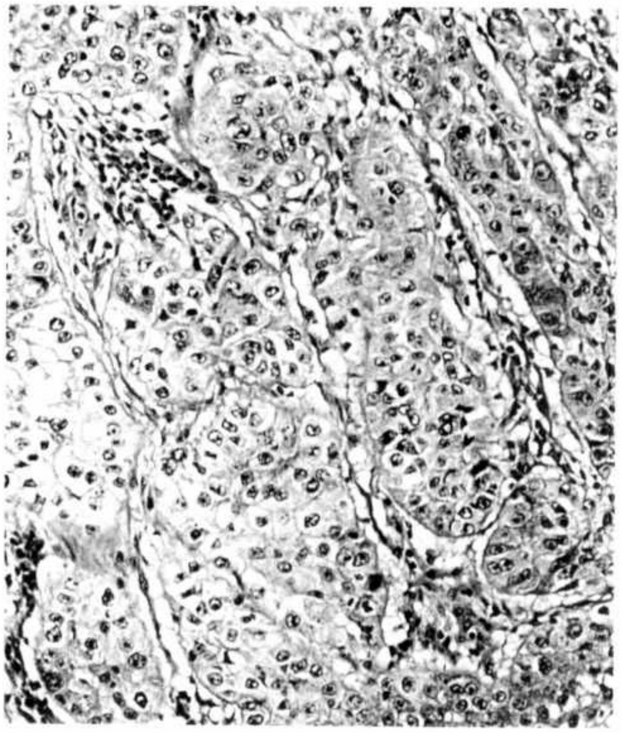

Figure 2. Histological appearance of the tumour composed of lobules of cells with abundant foamy cytoplasm. There is considerable variation in nuclear size and shape, and several mitotic figures are seen.

was negative. Fat stains could not be performed as all the material had been embedded in paraffin. The tumour was unconnected with the epidermis and there was no evidence of basal cell carcinoma-like change in any of the sections. On the basis of these findings a histological diagnosis of sebaceous gland carcinoma was made. Extensive investigations of the patient revealed no evidence of malignancy elsewhere in the body. He died 4 months later from bronchopneumonia; a post-mortem examination was not performed.

Discussion

Sebaceous carcinoma is an uncommon neoplasm most frequently seen on the eyelids where it accounts for 1% of malignant tumours at this site.[1] Extra-ocular sebaceous carcinomas appear to arise almost exclusively in middle-aged or elderly patients with a peak in the seventh decade of life. Males are affected twice as frequently as females. The majority of the lesions arise on the head and neck where they usually appear as a nodule which may ulcerate.[2] A patient with sebaceous carcinoma of the nose, said clinically to resemble a rhinophyma, has been previously described.[3] Our patient had a rhinophyma for many years, but it is significant that he had noticed progressive enlargement over the 12 months prior to his presentation. This enlargement was clearly due to the growing tumour. There are no specific markers for sebaceous carcinoma and the diagnosis is made by its distinguishing features of sebaceous cells with foamy cytoplasm and the cellular features of malignancy. The differential diagnosis includes sebaceous adenoma, basal cell carcinoma with sebaceous differentiation and metastatic clear cell tumour. Many different tumours may produce 'clear cell' metastases, the most common being renal and prostatic carcinomas. True sebaceous carcinomas have the ability to metastasize and should be excised with a wide margin. The paucity of previous reports suggests that the development of sebaceous carcinoma in rhinophyma tissue is purely a random occurrence.

Rapid enlargement of a rhinophyma should be considered as a sign of possible tumour development within the tissue.

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Malignant seeding of needle aspiration tract: a rare complication

J.M.NANKHONYA AND H.D.ZAKHOUR*

*Departments of Medicine/Geriatrics and *Pathology, Arrowe Park Hospital, Wirral L49 5PE, U.K.*

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Summary A case is reported of implantation metastases occurring at pleural aspiration sites in a 70-year-old man with pulmonary adenocarcinoma.

Case report

A 70-year-old retired seaman without previous respiratory symptoms presented in May 1989 with a 4-month history of progressive dyspnoea. There was no history of recent trauma and his weight had been steady. He had given up smoking 30 years previously. He also had well-controlled Type 2 diabetes.

Clinical examination was consistent with a massive right pleural effusion which was confirmed by a chest X-ray. Therapeutic and diagnostic pleural aspirations as well as a pleural biopsy were conducted using a 16-gauge Trucut needle. The cytology of the pleural exudate showed adenocarcinoma cells. Histology of the core of tissue removed showed diffuse infiltration by a well-differentiated adenocarcinoma. Histochemical stains showed a moderate amount of mucin in the cytoplasm of the cells.

Four months later he was readmitted for a further pleural aspiration. At that time he was cachectic and there were two well-circumscribed skin nodules that measured 1·5–2 cm in diameter at the previous aspiration sites (Fig. 1). Needle aspiration of the nodules confirmed that they were adenocarcinoma deposits (Fig. 2). The condition of the patient rapidly deteriorated and he died. Post-mortem examination was not carried out.

Discussion

Percutaneous needle biopsy of accessible lesions with or without imaging has become widely practised. The main indication is suspected malignancy and positive yields of up to 93% have been reported.[1] The probability of a positive biopsy result strongly correlates with the size of the lesion as well as experience in cytological processing.[1–4] Needle aspiration/biopsy is of proven superiority to open lung biopsy in that it is much easier and safer as well as being less expensive. This procedure can be done on day cases and can be readily repeated and may be used on multiple lesions even in frail patients.[1]

An uncommon complication of percutaneous needle aspiration (excepting malignant mesothelioma) is seed-

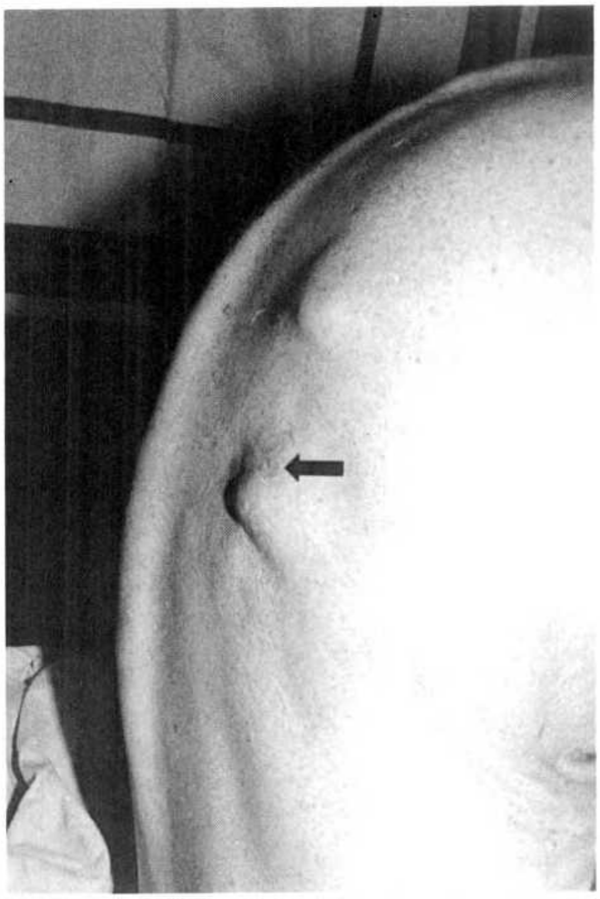

Figure 1. Subcutaneous nodules on pleural biopsy/aspiration sites on posterior chest wall. Scarring caused by needle punctures (↑).

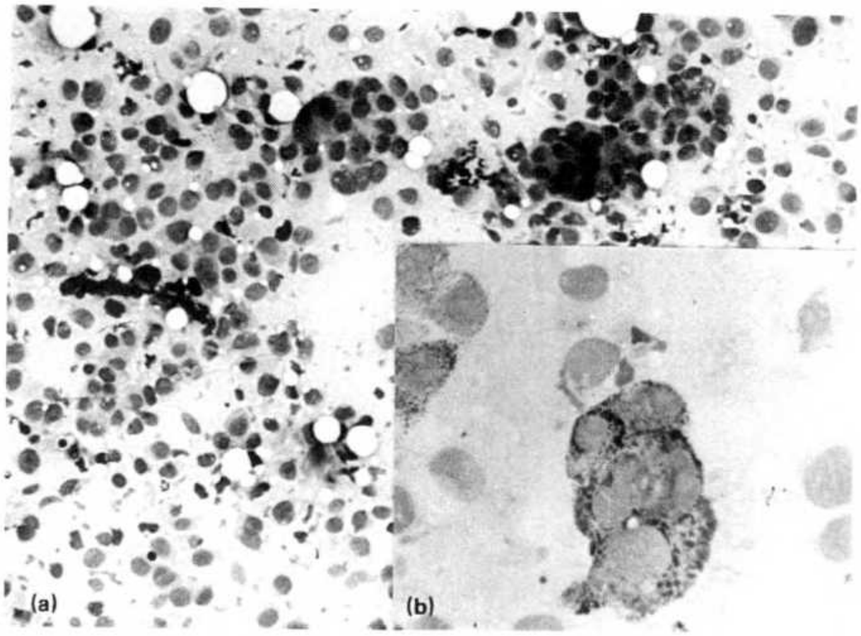

Figure 2. (a) Needle aspirate of skin nodules showing many malignant cells, some arranged in clusters (haematoxylin and eosin, × 130). (b) Inset: positive staining of cells for mucin (PAS, × 324).

ing of malignant cells along the biopsy tract. This is recognized by the presence of tumour cells in the skin or subcutaneous tissue at the site of the aspiration/biopsy. One case of implantation metastasis has been reported out of 1264 positive malignant percutaneous lung biopsies in 2726 subjects in whom 5300 procedures had been carried out over a 14-year period.[3] In a survey involving 1562 needle biopsies no cases of implantation metastases were identified.[5] The risk of tumour dissemination through the lymphatics and small veins was also discounted by Sinner[6] when he recorded no adverse effect on survival in a cohort with pulmonary neoplasia who had transthoracic needle biopsies.

Implantation of tumour cells along the needle tract is mainly related to the size of the needle and is very rare when those of 20–23 gauge (OD 0·6–0·9 mm) are used.[1,2] This is probably because larger bore needles allow aspiration of sizeable stroma fragments which may play a crucial role in the survival of cancer cells.[7] A noteable complication of percutaneous needle biopsy is pneumothorax whose pickup rate increases if a repeat chest X-ray is done 24 h after the procedure.[8] Haemorrhage, pleural effusion, haemoptysis, sepsis, air embolism and death are unusual. Overall complications will depend on needle size, number of biopsies taken per sitting, operator's experience and co-existence of respiratory disease, especially emphysema.

The use of needle biopsy as a diagnostic method will no doubt increase with the need for histological diagnosis before thoracotomy. Although needle tract seeding of malignancy is reportedly rare, we are in agreement with Denton *et al.*[9] who recommmend reporting this complication to establish its true incidence.

Acknowledgment

We thank Dr S.S.Garewal for permission to report this case and Dr J.C.Delaney for reading the manuscript.

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Malignant transformation of a clonal seborrhoeic keratosis

A.P.GALLIMORE

Department of Histopathology, The Royal Postgraduate Medical School, Du Cane Road, London W12 OHS, U.K.

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Summary A case is presented of malignant transformation, with early dermal invasion, within a clonal seborrhoeic keratosis situated in non-exposed skin.

Seborrhoeic keratoses are common skin lesions that arise predominantly on the face and trunk, but also on the extremities, in middle age and beyond. They may occur singly, but are more often multiple. The great majority are benign and have no malignant potential, but such transformation is probably not uncommon.[1] It is usually manifest as Bowenoid change within an acanthotic or hyperkeratotic lesion located in sun-exposed skin. The present case is unusual in that it is an example of malignant transformation of a clonal seborrhoeic keratosis situated on the abdominal wall, protected from the sun, with superficial dermal invasion.

Case report

A skin lesion measuring $3 \times 2 \times 0{\cdot}5$ cm was removed from the anterior abdominal wall of a 73-year-old Caucasian female. The lesion had been present for several months, but had recently increased in size. It consisted of an ill-defined, irregular elevation of the skin with multiple nodular areas. A few of the nodules had a verrucous appearance. The clinical impression was of squamous cell carcinoma.

Histologically, the skin had an irregular papillomatous surface with areas of hyperkeratosis and acanthosis alternating with relatively flat areas (Fig. 1). Well defined nests of small, uniform, basaloid cells were present within the acanthotic epidermis. These cells showed no atypical features and were typical of the clonal variant of seborrhoeic keratosis (Fig. 2). The dermis displayed only mild chronic inflammation and no solar damage. Elsewhere there was a larger, nodular lesion surmounted by a parakeratotic crust in which there was transformation to a squamous cell carcinoma (Fig. 3). The neoplastic cells exhibited marked nuclear pleomorphism, single-cell degeneration and abnormal mitoses (Fig. 4). There was a small focus of dermal invasion (Fig. 5). There was more florid chronic inflam-

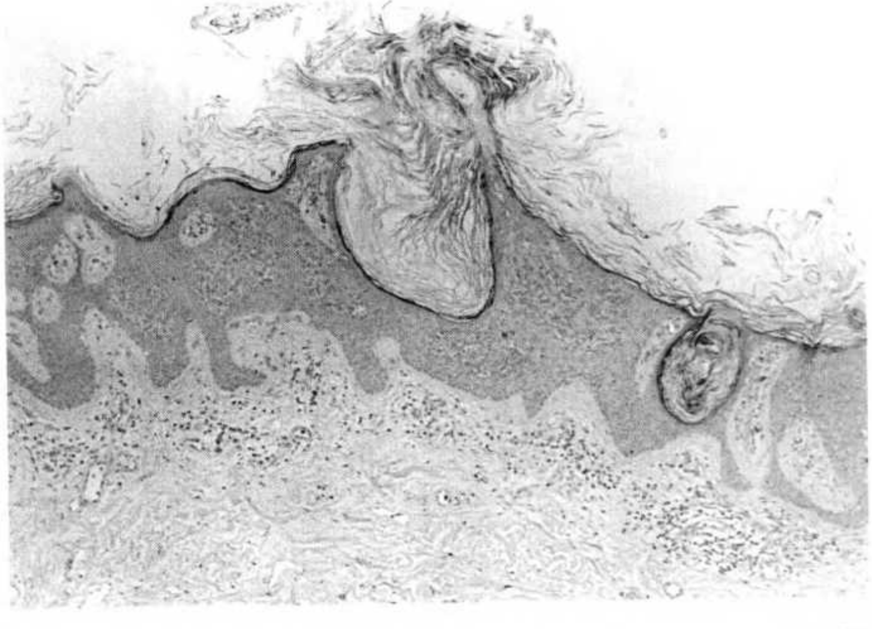

Figure 1. Areas of papillomatosis, acanthosis and hyperkeratosis are present, along with keratin-filled pseudocysts (Haematoxylin and eosin, × 20).

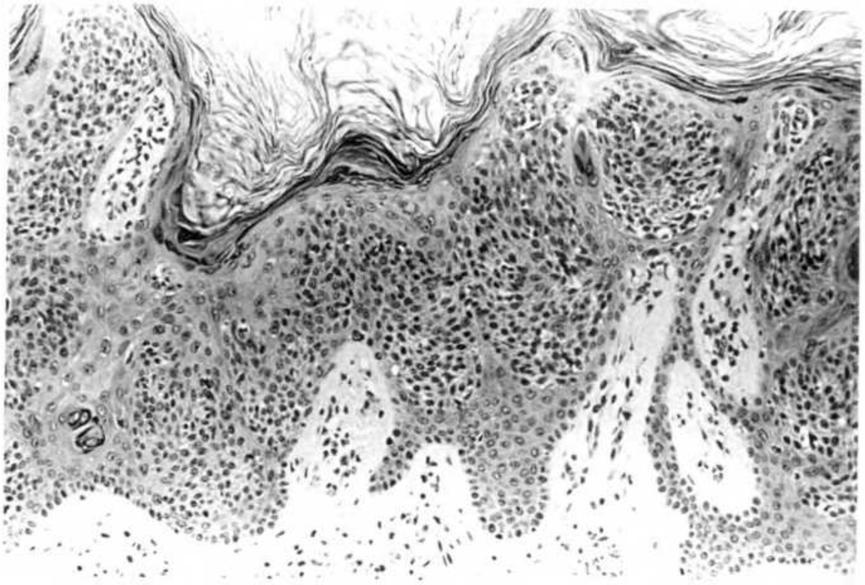

Figure 2. Nests of small, basaloid cells are seen within the epidermis (Haematoxylin and eosin, × 100).

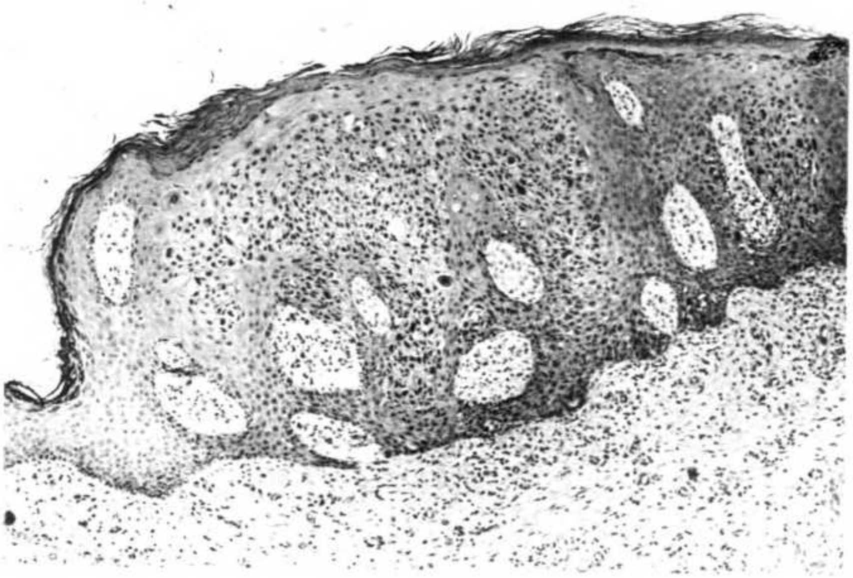

Figure 3. A single nodule shows transformation to a squamous cell carcinoma (Haematoxylin and eosin, × 100).

mation and capillary proliferation within the subjacent dermis, but no significant solar damage. Immunoperoxidase staining using a cytokeratin monoclonal antibody (BioGenex Laboratories H171-5c pancytokeratin) demonstrated reduced reactivity in both the cells of the basaloid clones and in the area of malignant change (Fig. 6). The intensity of reaction in the basaloid nests was similar to that of the subjacent basal cells. The lesion was completely excised and there has been no recurrence and no new lesions over a period of 12 months.

Discussion

Seborrhoeic keratoses are benign cutaneous neoplasms composed of variable proportions of basaloid and squamoid epidermal cells. Six histological patterns are recognized: acanthotic, hyperkeratotic, irritated, melanoacanthoma, clonal and reticulated. The latter two are relatively uncommon. All types exhibit varying degrees of hyperkeratosis, acanthosis and papillomatosis. In the past the nests of basaloid cells that are characteristic of the clonal variant have been interpreted as examples of intraepithelial epithelioma of Jadassohn, and was the commonest of several lesions to be viewed in this way.[2] The term clonal seborrhoeic keratosis was first used by Okun and Edelstein for such lesions with intraepidermal nests of basaloid cells.[3] The most important non-pigmented premalignant skin lesions are actinic

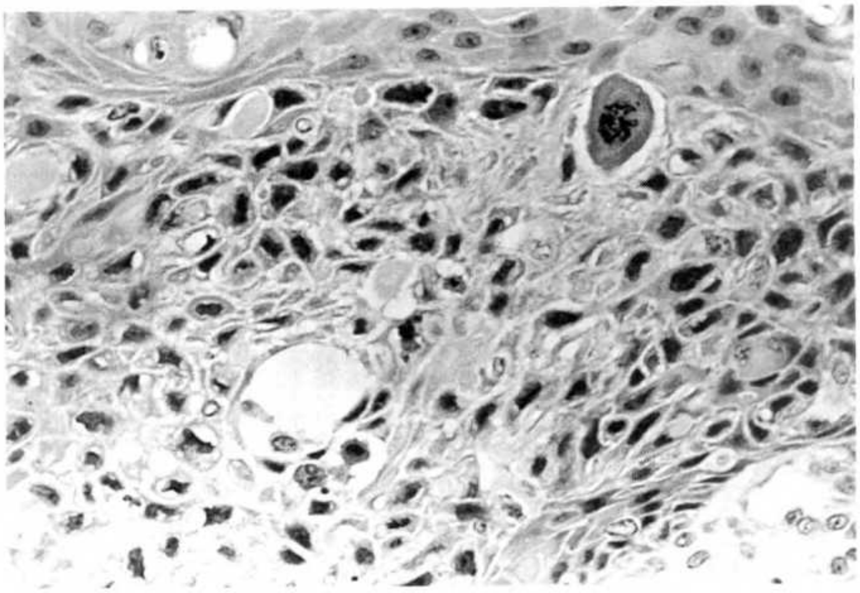

Figure 4. The neoplastic cells exhibit nuclear pleomorphism, hyperchromasia and mitotic activity (Haematoxylin and eosin, × 250).

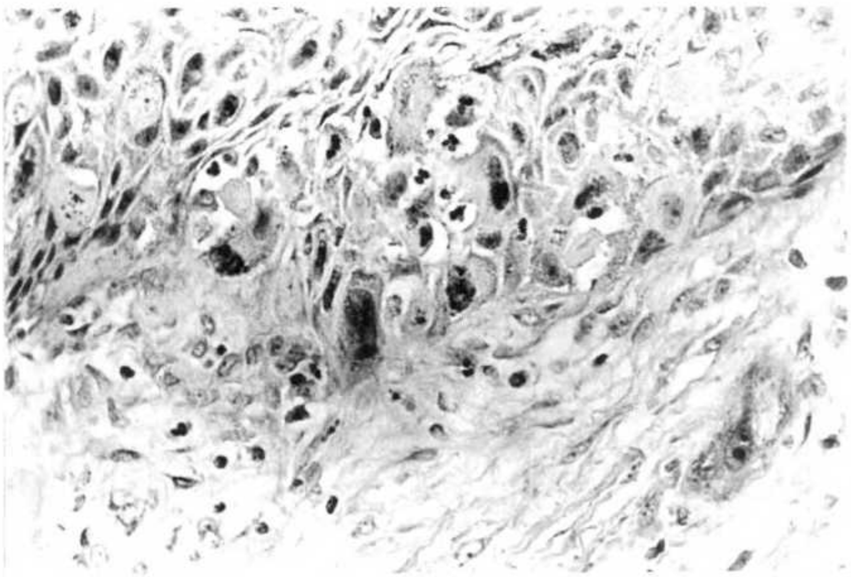

Figure 5. Neoplastic squamous cells are present within the superficial dermis (Haematoxylin and eosin, × 250).

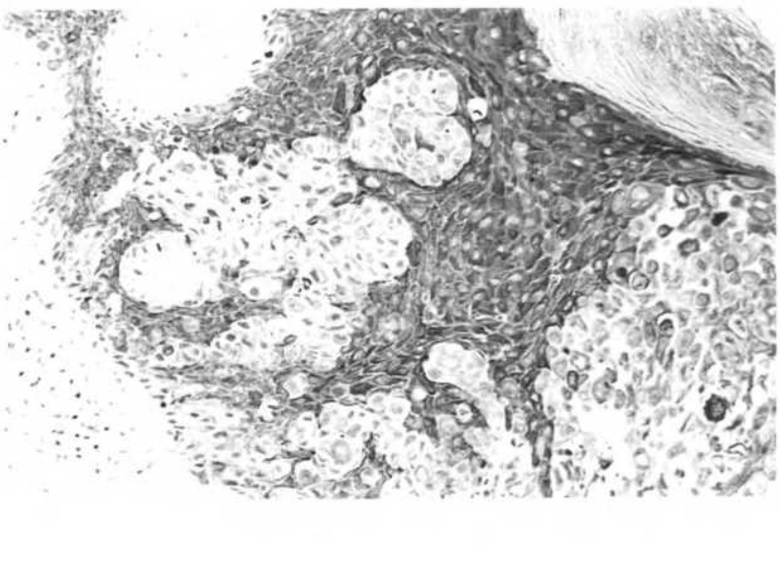

Figure 6. The neoplastic cells show weak staining compared to the adjacent epidermal cells (× 250).

keratosis, Bowen's disease and keratoacanthoma.[4] Malignant transformation occurring within seborrhoeic keratoses has been reported in the literature on several occasions[5–7] and is probably a common event.[1] The majority of cases are in hyperkeratotic or acanthotic lesions present on sun-exposed sites, and solar damage may be important in the pathogenesis of this transformation.[5] Most are examples of squamous carcinoma *in situ*, with little disturbance in the overall architecture of the lesion, but a papillated lesion has been described.[8] Basal cell carcinoma arising from a seborrhoeic keratosis is more unusual.[9] Invasive squamous carcinoma is rare, but Christeler and Delacretaz described a case in which there were regional lymph node metastases.[10] Definite malignant transformation within a clonal seborrhoeic keratosis has not been documented previously, although both atypia and maturation were provoked in such a lesion following biopsy.[11] The present case is distinctly unusual as there is evidence of superficial dermal invasion, and the lesion arose in non-exposed skin and had not been traumatized. This report highlights the possibility of malignant transformation in what are generally considered to be benign lesions, and that this may occur in the absence of sun exposure.

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Adult lipophagic atrophic panniculitis

I.J.UMBERT AND R.K.WINKELMANN
Department of Dermatology, Mayo Clinic and Mayo Foundation, Rochester, MN, U.S.A.

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Summary We report two patients with chronic, recurrent, nodular panniculitis lesions that later developed areas of lipoatrophy. Histologically, there was a consistent lobular lipophagic replacement of fat cells with lipophagic giant cells. The clinical appearance was that of tender, erythematous, superficial or subcutaneous, symmetrical nodules and plaques of 1–2 weeks' duration. The lesions could occur with episodes of fever. One patient had hepatomegaly and the other had an increased sedimentation rate and leucocytosis.

The histology and the clinical pattern of the panniculitus syndrome resembled those of lipophagic lipoatrophy of childhood. This is a panniculitis of unknown cause in which the principal inflammatory cell response in the subcutaneous tissue is the macrophage.

Descriptions of Weber–Christian panniculitis have included the histology of lipophagic replacement of fat lobules since the early report by Weber.[1] Subsequently, lipophagic replacement of fat has been used to define this syndrome as a pathological entity.[2] Pfeifer[3] described a 23-year-old woman with primary lobular panniculitis, lipoatrophy with lipophagia, and giant cells. The locations of the lesions and muscle weakness were somewhat atypical and also raised the question of panniculitis with connective tissue disease.

Lipophagia has recently been described as the primary pathological process in a rare lipoatrophic syndrome of panniculitis in childhood.[4] All of these cases had been called Weber–Christian disease or Rothman–Makai syndrome.[5–18] Winkelmann *et al.*[4] renamed these cases lipophagic lipoatrophy of childhood, recognizing that the cases began with erythematous nodules of short duration, with a recurrent course and lipophagic giant cell replacement of fat, and with residual lipoatrophic lesions.

We have studied two adult patients with a similar syndrome. All the features of the childhood phenomenon were present, and we wish to document this as a syndrome of panniculitis that can occur at any age. In our experience this condition is unique in comparison to other forms of delineated panniculitis as in connective tissue disease, pancreatic disease, or α_1-antitrypsin deficiency.

Case reports

Case 1

A 47-year-old woman was first seen in our department in February 1986. In November 1985 diffuse tender erythematous subcutaneous nodules and plaques had developed on her right thigh and buttocks. No adenopathy and visceromegaly were found. Investigations included a normal ultrasound of the pancreas and normal serum amylase and lipase levels, negative antinuclear antibody and normal levels of complement and cryoglobulins. Cultures of the lesions for bacteria, mycobacteria, and fungi were sterile. The level of α_1-antitrypsin was normal. Other investigations revealed that her sedimentation rate was 8 mm/h (Westergren method) and the haemoglobin level was 14 g/dl. Her leucocyte count was $7 \cdot 8 \times 10^9$/l, with a normal differential. Total thyroxine, aspartate aminotransferase, cholesterol, and triglyceride levels and blood chemistry values were normal.

The patient was started on dapsone, 50–100 mg daily and showed some resolution of her lesions. There were some broad atrophic depressions on the buttocks, thigh, and right arm at the sites of previous lesions (Figs 1 and 2).

In March 1989 recurrent red nodules on both legs developed, and 1 day later fever and chills developed. She was treated with dapsone and then sulphapyridine until progressive weakness and shortness of breath developed. Chest X-rays showed a diffuse bilateral infiltrate involving interstitial and alveolar components. These changes were consistent with a hypersensitivity pneumonitis,

Correspondence: Professor R.K.Winkelmann, Department of Dermatology, Mayo Clinic, 200 First Street SW, Rochester, MN 55905, U.S.A.

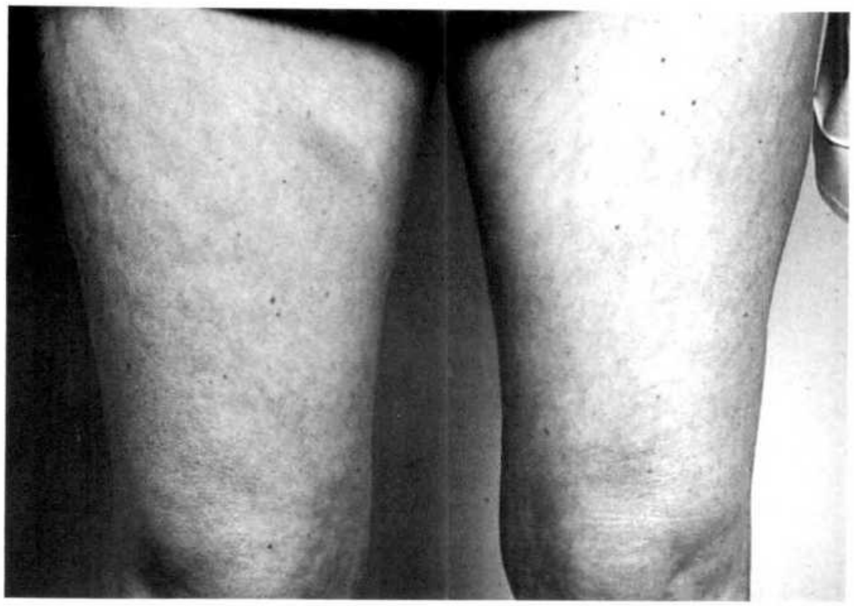

Figure 1. (Case 1). Inflammatory nodule on anterior right thigh. April 1989.

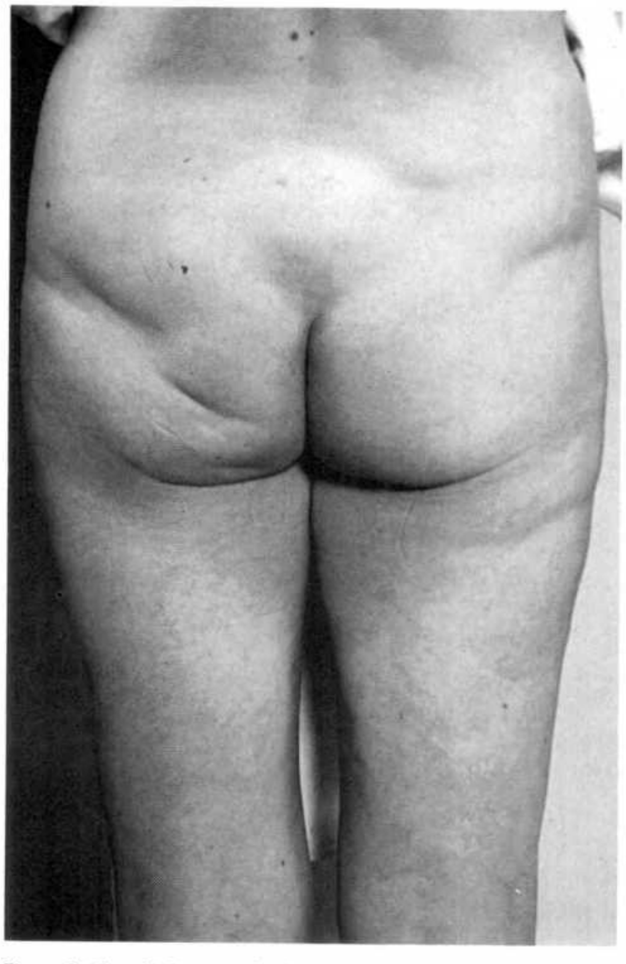

Figure 2. (Case 1). Lipoatrophic lesions of buttocks in areas of previous lipophagic panniculitis. April 1989.

possibly due to sulphapyridine. The patient discontinued the sulphapyridine, with improvement of the clinical signs and symptoms and clearing of the skin lesions. The patient had no skin lesions 6 months later, except for the previously developed lipoatrophy of the buttocks.

Case 2

A 57-year-old woman was first seen in our department in July 1980. Since January she had had very painful indurated subcutaneous nodules and plaques over the chest, back, abdomen, legs, arms, and breasts. These occurred in crops, and the lesions lasted for 7–10 days. She also had a temperature of up to 38·3°C and chills for 2–3 days, with generalized malaise accompanying the eruption. On two occasions the lesions spontaneously drained an oily material and resolved. Several lesions left pigmented residual macules. Physical examination was negative except for hepatomegaly. She had had an intermittent arthritis of the right ankle with swelling and tenderness and increased warmth since 1980.

Laboratory evaluation included negative tests with purified protein derivative, a negative rheumatoid factor, and normal blood cell counts except for occasions when the leucocyte count was increased up to 17×10^9/l. She had normal blood chemistry values. The blood sedimentation rate varied from 30 to 120 mm/h. There were normal throat and sputum cultures and samples of sputum were negative for acid-fast bacilli and fungi. The chest X-ray was normal. She also had normal antinuclear antibodies, normal C3, and normal blood cultures. X-rays of the right ankle showed osteoporosis, and the joint spaces were narrowed. Cultures of the tissue biopsy specimens were negative for routine and anaerobic organisms, mycobacteria, and fungi. She had normal serum protein electrophoresis except for a mild elevation of gamma globulin levels. Studies of the pancreas and gastrointestinal tract were negative. The levels of α_1-antitrypsin, serum lipase, and amylase were normal on three occasions. Urine amylase was normal. Creatine phosphokinase muscle enzyme determinations were normal.

She had been treated with diuretics and hydralazine in the past 5 years for hypertension.

The patient was treated with systemic prednisone in a dosage of up to 200 mg/day. Later, antimalarial drugs were added but with no improvement of the clinical picture. Mild atrophy of the panniculus of the left calf subsequently developed. Subsequently, the patient has presented with several episodes of painful joint swellings and multiple tender subcutaneous nodules associated with cutaneous erythema and fever.

Histology

The patients had lobular and septal lipophagic panniculitis. The infiltrate was predominantly histiocytic, with a distinctive predominance of foamy histiocytes (lipophages) and Touton-like lipophages that involved the subcutaneous fat and periappendageal fat (Fig. 3). These cells were confluent with a central mass of nuclei within the lipophagic giant cell cytoplasm (Fig. 4). The infiltrate also contained lymphocytes, a single large follicular-like germinal centre (Case 1), neutrophils, and plasma cells. Numerous eosinophils were present in the infiltrate in Case 1 and also were seen within the granuloma. There was a superficial and deep perivascular infiltrate in the dermis composed mostly of lymphocytes and histiocytes. In Cases 1 and 2, the inflammatory infiltrate also surrounded the adnexal structures. The blood vessels were uninvolved. Focal fat necrosis was observed in Case 2. Two biopsy specimens of Case 1 from 1986 and 1989 showed an identical pathological picture with no substantial variation.

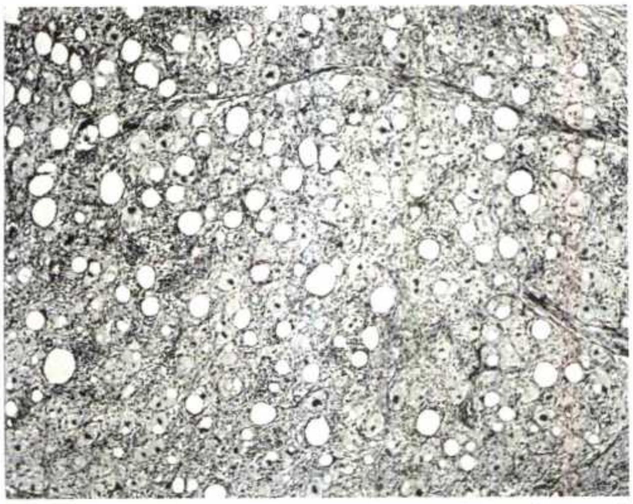

Figure 3. (Case 1). Complete lobular replacement by lipophages and lipophagic giant cells. (Haematoxylin and eosin: × 44.)

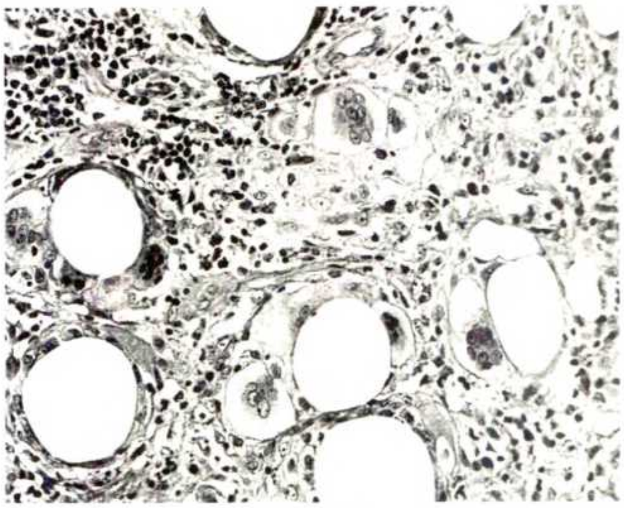

Figure 4. (Case 1). Mixture of lymphocytes and lipophages replacing and surrounding fat cells. Lipophage giant cells have characteristic central cluster of nuclei. (Haematoxylin and eosin: × 276.)

Macrocyst and microcyst formation were commonly seen. Extensive fibrosis involving the subcutaneous tissue was evident in Case 1. Special stains for bacteria, fungi, and mycobacteria were negative. Polarization microscopy failed to reveal any foreign material. Direct immunofluorescence of involved skin in Case 1 showed deposition of fibrinogen within vessels and IgM, IgA, and C3-containing inflammatory cells in the fascia.

Monoclonal antibody studies of the tissue of Case 1 revealed 40% of cells positive for CD14 (Leu-M3), a monocyte antibody. Diffuse staining of small round cells was present through the fat lobules, and 40% of the cells were positive for CD5 (Leu 1) and CD3 (Leu 4) pan-T cell markers. Some CD8 (Leu 2a) positive T-suppressor cells were present in the lipophagic lobules. CD4 (Leu 3a) positive T-helper cells were diffused through the infiltrate, in a concentration of 30–40%. Rare B-cell nodules were identified by CD22 (Leu 14). Staining for CD1 (OKT6) was negative in the inflammatory fat but was

Table 1. Cases of adult lipophagic lipoatrophy

Patient		Lesion		
Age (years)	Sex	Location	Duration	Reference
23	F	Face, breasts, arms	Recurrent	Pfeifer, 1892[3]
47	F	Thigh	2 weeks	Kooij, 1950[19]
47	F	Thigh, buttocks, legs	Recurrent	Umbert, Winkelmann, 1990
57	F	Trunk, legs	Recurrent	

markedly positive for dendritic cells in the epidermis and hair follicles. CD38 (Leu 8) staining showed focal rare isolated cells. Staining for CD7 (Leu 9) was negative.

Discussion

We have found only one similar case of an adult patient with multiple subcutaneous lipophagic panniculitis nodules of the thigh lasting 2 weeks and leaving lipoatrophy.[19] The histopathology of this case was massive lipophage and giant cell lobular panniculitis. This case is included with our two cases of lipophagic and lipoatrophic panniculitis of the adult (Table 1). This is an extremely rare syndrome in adults and could be confused with other diseases.

Weber[1] described a patient with tuberculosis and a classical erythema nodosum syndrome. The histopathology was similar to our present cases of lipophagic lobular panniculitis with lipophagic giant cells. His case is one of the index cases included in the collective description of 'Weber–Christian disease'. The childhood cases of Cummins and Lever[6] also showed lipophagic panniculitis with lipoatrophy, and were published as Weber–Christian disease; as were additional paediatric cases. It has been pointed out that cases of erythema nodosum may show lipophagia[19,20] which is also part of other disease states of the fat.

In 1913, Galloway[21] described large lipophages in a patient with erythema induratum. Several cases of erythema nodosum described as Weber–Christian disease demonstrated lipophages with some giant cells as common findings.[22] Lipophages have also been found in foreign body reactions,[23] lipomembranous panniculitis,[24] mesenteric panniculitis,[25] fat necrosis of the breast,[26,27] and re-excision of surgical specimens[28] (Table 2).

Recently, Winkelmann *et al.*[4] described three patients with an atrophic lipogranulomatous lipoatrophy of childhood and compared these cases with 14 similar cases from the literature. Four additional cases have subsequently been described by Larbre *et al.*[18] In these cases there was a consistent replacement of fat lobules by lipophagic histiocytes and lipophagic giant cells. The disease was commonly recurrent, and erythematous or deep nodular lesions of panniculitis could be present. Fever was frequently present. The lesions would last for 3 weeks or more. On occasions, large plaque lesions would coalesce, persist, and extend to cover extensive areas. Five patients had evidence of liver disease as indicated by hepatomegaly; changes in the liver enzymes, or on liver biopsy. One of our two current adult patients had hepatomegaly.

Table 2. Lipophagic panniculitis

Primary
- Acute, benign—children
- Recurrent lipoatrophic—children
- Acute or recurrent—adults

Secondary
- Erythema nodosum
- Erythema induratum
- Lipomembranous panniculitis
- Mesenteric panniculitis
- Fat necrosis of the breast
- Foreign body reaction
- Surgical re-excision

The evolution of the lipoatrophy in the children gave rise to the 'hewn extremity sign'. This is similar to the large cupuliform atrophy seen in our adult patients with this same histology.

A lipophagic panniculitis of benign course and rapid self-healing lesions has been described by Royer *et al.*,[29] Turpin and Bocquet,[30] and Labeille *et al.*[31] This panniculitis presents in children with or without fever or respiratory tract infection. These lesions heal spontaneously in 7–21 days and no treatment is necessary. The histopathology is of panlobular lipophagic panniculitis with lipophagic giant cells. There is no lipoatrophy or other residual of the lesions.

We conclude from these reports that the pathology of lipophagia can be primary or secondary. In primary lipophagia, no prior disease or injury to the subcutaneous tissue can be identified, and the syndromes listed in Table 2 as primary are idiopathic panniculitis with lipophagia. The secondary responses include responses to known injury or inflammation. Some of these primary reactions may be immunological, and some may be physical or toxic. It seems probable that the typical response occurs from some monokine stimulus released from the inflammation or produced by some special host factor developing from the diffuse macrophage/lipophage panniculitis. The monokine may originate from the T cells identified in Case 1.

The patients described in this paper represent an extremely rare variation of primary lipophagia that developed lipoatrophy. The histopathology is characteristic. The response to treatment is variable, but corticosteroids used early can prevent much of the lipoatrophy and the inflammatory signs and symptoms. It seems to us that this is equivalent to the disease as observed in children, and on that basis, we suggest that in these patients a complete investigation of liver function should be performed.

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Bullous pemphigoid in a patient with a C4 deficiency

S.SHIRAISHI, T.IIO, Y.SHIRAKATA, K.SAYAMA, H.NISHIMUKAI* AND Y.MIKI

*Department of Dermatology and *Legal Medicine, University of Ehime School of Medicine, Ehime, Japan*

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Summary A 53-year-old man with bullous pemphigoid with an extremely low level of C4 in the serum is reported.

Bullous pemphigoid (BP) is an autoimmune blistering disorder with circulating antibodies that are reactive to an antigen in the basement membrane zone (BMZ). The immune complexes are deposited and activate complement with the subsequent migration of inflammatory cells. Direct immunofluorescence shows deposition of IgG and complement at the BMZ in both involved and perilesional areas of skin. We report a patient with bullous pemphigoid who had a C4 deficiency state.

Case report

In July 1984, a 53-year-old Japanese man was seen with a 6-month history of a generalized erythematous eruption with tense bullae. The blisters appeared on the thigh and neck and subsequently spread to involve the entire body with severe itching and the patient was pyrexial and oliguric. He was treated with oral prednisolone 75 mg daily, but continued to have blistering of the skin. On examination he appeared ill with Cushingnoid signs and had muscle weakness. There was a diffuse erythema over much of the skin surface with many tense blisters and erosions (Fig. 1). The lips and buccal mucous membrane were also affected. The serum IgE was elevated at over 4000 IU/ml and the total complement level (CH50) was reduced to below 4 U/ml. A skin biopsy showed a subepidermal blister with an inflammatory cell infiltrate in the dermis consisting of eosinophils, lymphocytes and neutrophils. Direct immunofluorescence (DIF) of the perilesional skin showed a linear deposition of IgG and C3 at the BMZ. There was no deposition of IgA or IgM. Indirect immunofluorescence using guinea-pig mucous membrane as the substrate showed a circulating antibody in the serum against BMZ with a titre of 1:64. The BP autoantibody in the serum was detected using indirect IF on normal human skin sections with monoclonal anti-human IgG and anti-IgG subclass antibodies (AMAC Inc, Westbrook, ME, U.S.A.). Subtyping of IgG showed only IgG4 subclass. IgG1, 2 and 3 subclasses were not found.

The patient was commenced on oral betamethasone 6

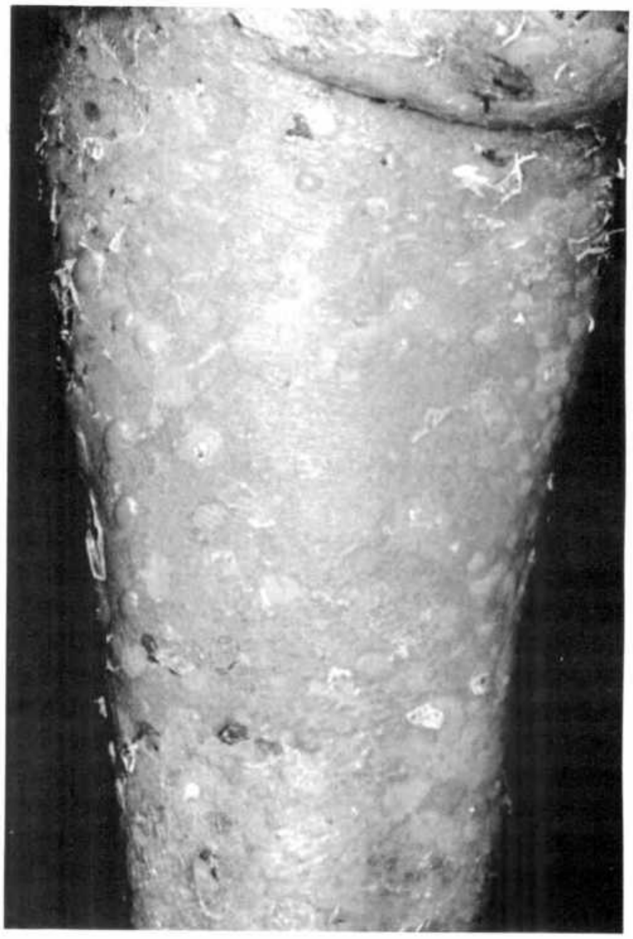

Figure 1. Tense blisters and erosions on the thigh.

Correspondence: Dr S.Shiraishi, Department of Dermatology, University of Ehime School of Medicine, Shigenobucho, Onsengun, Ehime 791-02, Japan.

Table 1. Haemolytic complement studies

	CH50 (units)	SFU/ml*								
		C1	C2	C3	C4	C5	C6	C7	C8	C9
Patient	3·4	1200	370	23,000	3·4	590,000	22,000	9000	12,000	3100
Son	37·2	93,000	1400	20,000	25,000	122,000	14,000	7000	5200	4900
Daughter	39·5	103,000	980	30,000	22,000	127,000	19,000	8400	4800	4900
NHS-P	37·8	15,000	1200	27,000	45,000	130,000	18,000	8100	4600	4800

*Haemolytic activities of C1-C9 were expressed as site-forming units (SFU/ml). NHS-P, normal human serum pool.

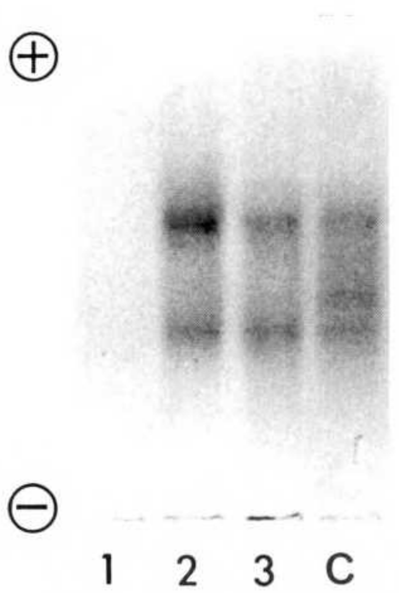

Figure 2. Patterns of C4: 1, patient; 2, daughter (C4 A3 B1); 3, son (C4 A3 B1); C, control (C4 A3 B1).

mg daily and within 7 days there was a marked improvement. The dose was gradually reduced to 1·5 mg daily. By the 32nd day circulating anti-BMZ antibody was not detected and the blood eosinophilia decreased from 8·9 to 0·6%. The serum IgE level remained unchanged.

Analysis of the various components of complement was carried out on samples of blood taken from the patient and from other members of his family.[1–5] The results of CH50 and complement component determinations in the patient and in controls are shown in Table 1. In our patient, CH50 was reduced and the C4 level was extremely low at 0·0075% of the normal human serum pool. C1 and C2 levels were also reduced to 8 and 30·8%, respectively. The other complement components were within the normal range. In our patient the CH50 and C4 levels remained low even after 5 years when the patient had remained improved. The complement levels of the patient's son and daughter were normal. The concentrations of C3, C4 and C1 inhibitor in the patient were 120 mg (normal range 80–140 mg), 3·1 mg (20–40 mg) and 115% of the normal serum pool, respectively. Immunofixation electrophoresis of C4 in neuraminidase-treated plasma revealed no detectable band(s) while C4 A3 B1 phenotypes were found in the son and daughter (Fig. 2).

Normal human skin explants cultured for 24 h or 48 h with the patient's serum or with blister fluid developed deposits of IgG and C3 at the BMZ (Fig. 3).[6] There were no histological changes to suggest a dermal–epidermal split.

Discussion

Both IgG and C3 deposits at the BMZ have been reported in the majority of patients with bullous pemphigoid. The deposition of C3 results from circulating antibodies that activate complement via the classical or alternate pathways. The functional evidence for the activation of complement in BP lesions comes from the observation that levels of CH50 and individual complement components were reduced except for those of C8 and C9.[7] In our patient, CH50 and C4 levels were reduced with very low levels of C4 that persisted despite clinical improvement. This deficiency of C4 was not apparently inherited either by his son or daughter.

The fourth component of complement is composed of two highly homologous glycoproteins derived from two distinct structural genes; C4A and C4B. Partial C4 deficiency appears to be a common risk factor for the development of systemic lupus erythematosus, the complete C4A deficiency being present in 10–15% of patients with this disease.[8] However, there has been no report of C4 deficiency associated with bullous pemphigoid.

An *in-vitro* model of bullous pemphigoid has been

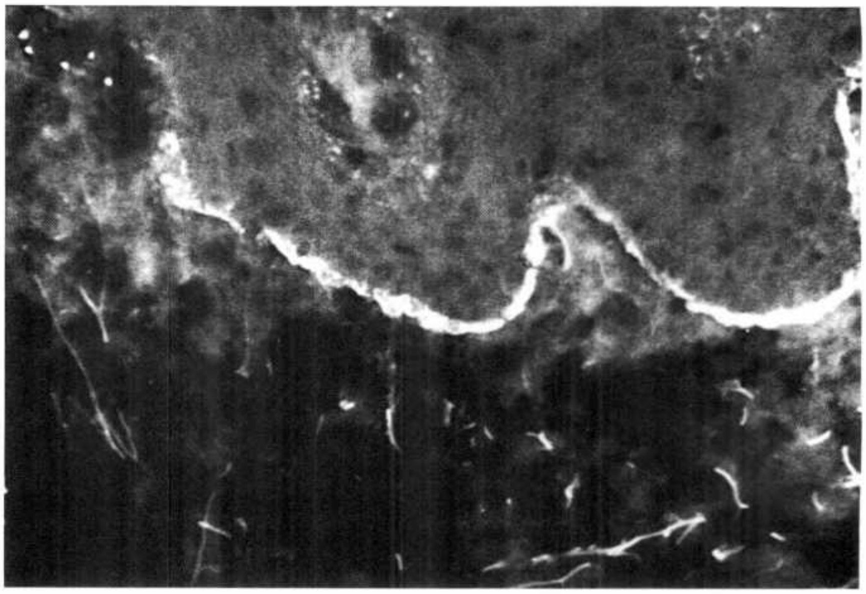

Figure 3. Direct immunofluorescence of normal skin explant cultured for 24 h with serum from patient showing deposits of C3 at BMZ.

developed by Naito *et al.*[9] When normal human skin was cultured with sera, IgG fraction or blister fluid from a patient with BP, antibody binding was observed using an immunofluorescent technique. Dermal–epidermal separation developed only in the skin explants cultured with blister fluid. When a high titre of a partially purified IgG fraction from a patient with BP was injected into guinea-pigs in which C3 was inactivated with cobra venom factor, dermal–epidermal separation was inhibited *in vivo*. Most, but not all, C4-deficient guinea-pigs demonstrated dermal–epidermal separation after the injection of the IgG fraction. These results suggest that the alternate pathway of complement is necessary for the separation that occurs at the BMZ.[10]

The role of complement as a direct mediator in the early events of blister formation in BP has recently been questioned. In our patient contrary to a previous report[11] subtyping of autoantibody showed only IgG4 and C3 deposition occurring both *in vivo* and *in vitro*. IgG4 is the least common of the four IgG subclasses and cannot activate complement via the classical pathway. The presence of the IgG4 subclass alone and the deposition of C3 at the BMZ in our C4-deficient patient, together with the results of the organ culture study, indicate that activation of complement occurred by the alternate pathway. However, it is possible that very small amounts of other IgG subclasses were present in the sera and induced the deposition of complement via the classical pathway.

Acknowledgment

This work was supported in part by a grant from the Japanese Ministry of Health and Welfare.

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British Journal of Dermatology (1991) **124**, 299–305.

Correspondence

Benign familial pemphigus responsive to cyclosporin, a possible role for cellular immunity in pathogenesis

SIR, Anecdotal reports of the beneficial response of benign familial pemphigus (Hailey–Hailey disease) to anti-inflammatory or immunosuppressive therapies such as steroids,[1] methotrexate,[2] dapsone[3] and PUVA[4] and its histological resemblance to pemphigus prompted us to use cyclosporin A (CyA) in two patients with this disease.

The first, a woman aged 43 years with a 33-year history of fissuring in the groins, erosions of the vulva and involvement of the sides of the neck and axillae, was diagnosed as suffering from Hailey–Hailey disease based on a strong family history (a sister, mother, seven maternal aunts and maternal uncle affected) and classical skin pathology. Over the previous 10 years she had only partial and temporary improvement with many therapies including oral and topical steroids, dapsone, methotrexate and PUVA.

The patient attended the skin department in January 1989 during an exacerbation with severe erosions of the groin. There was rapid improvement in the first week with CyA (5 mg/kg) together with augmentin to prevent secondary infection. The improvement was such that in spite of the dose of CyA being reduced to 3 mg/kg, the frequency, severity and duration of exacerbations was markedly reduced over the next 18 months. The patient judged this to be the most effective treatment she had received. Attempts to stop the CyA on two occasions resulted in rapid relapses within 1 week which resolved on resuming therapy with CyA alone. To reduce long-term toxicity, we attempted to transfer her onto topical therapy using 5% CyA with penetration enhancers (propylene glycol and azone) which have proven effective in enhancing CyA penetration *in vitro*.[5] This was applied to one groin whilst the vehicle without CyA was applied to the other but both sides relapsed within 3 days and, as the treatment was painful when applied to raw skin, it had to be stopped.

The second patient was the mother of Patient 1 (age 67) who requested the same treatment. She had more severe and extensive disease of 30 years duration under the breasts and in the axillae and groins, where wide areas were eroded, fissured and painful, restricting mobility. This severe exacerbation had been unremitting for 6 months whilst using clobetasol propionate 0·05% cream, but healed rapidly in 2 weeks after starting CyA 5 mg/kg. Because of nephrotoxicity and hypertension the dose was reduced to 1·4 mg/kg without relapse. Biopsies taken before treatment showed acantholysis and intraepidermal clefts which were still present to a lesser degree 1 month later when the lesions were clinically healed. Immunohistochemical staining revealed a marked infiltration of pan T (CD3$^+$) lymphocytes in the dermis of the untreated skin which was substantially reduced following treatment with CyA (Table 1, Fig. 1). This was reflected by reductions in the numbers of dermal T helper (CD4$^+$) and T suppressor/cytotoxic (CD8$^+$) cells in dermis and epidermis. The marked increase in epidermal Langerhans cell (CD1$^+$) numbers following CyA treatment probably accounts for the increase in epidermal CD4$^+$ lymphocytes as CD1$^+$ cells constitutively express CD4.[6] It appears that the activation status of lymphocytes was only partially inhi-

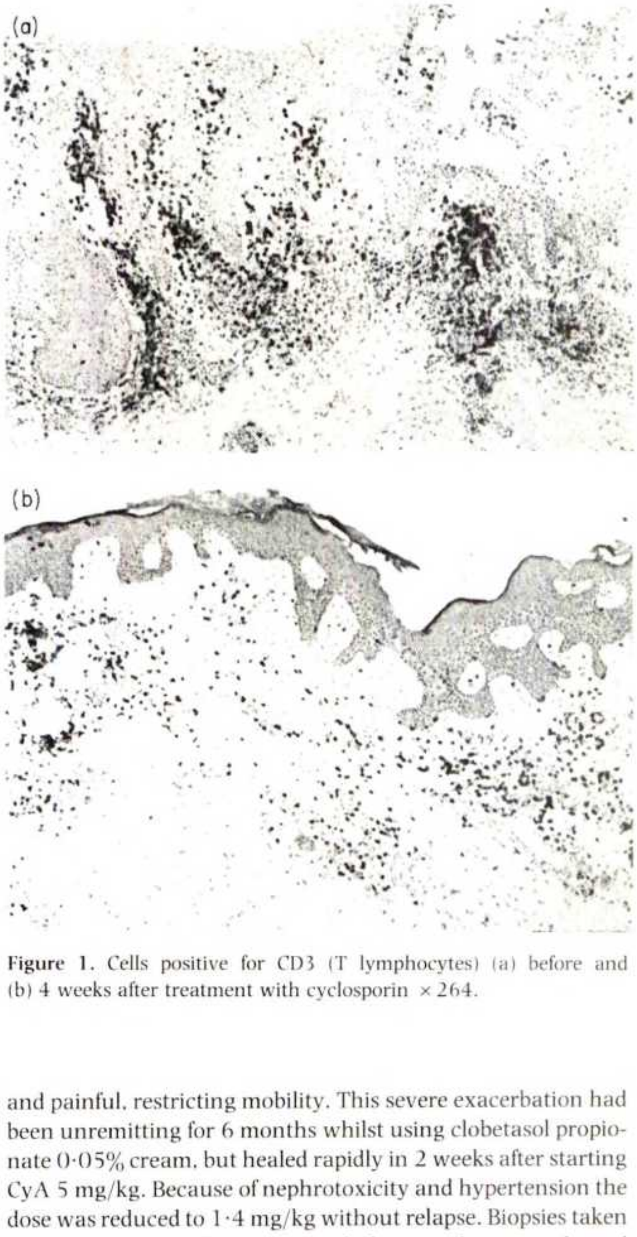

Figure 1. Cells positive for CD3 (T lymphocytes) (a) before and (b) 4 weeks after treatment with cyclosporin × 264.

Table 1. Positive staining cells/mm^2 before and after treatment with cyclosporin

	CD1	CD3	CD4	CD8	CD25	HLA-DR
Epidermis						
Week 0	5·2	12·1	7·4	5·2	2·1	12·5
Week 4	46·2	11·9	18·0	2·7	1·2	22·5
Dermis						
Week 0	0·5	163·4	89·1	39·3	15·2	62·9
Week 4	5·3	96·0	59·4	49·0	11·3	75·2

bited after 1 month of treatment with CyA as cellular expression of interleukin-2 receptor (CD25) was slightly decreased. The increase in HLA-DR may also be due to the increase in $CD1^+$ cells expressing HLA-DR.

The mode of action of cyclosporin in this condition is unknown but, whilst negative immunofluorescence makes a humoral autoimmune mechanism unlikely, the response to cyclosporin and the abundance of T lymphocytes in the dermal infiltrate (not previously reported in the literature) suggest that these cells play an important pathogenic role. The effects of CyA on the infiltrate are very similar to those demonstrated in psoriasis treated with CyA[7] with a diminution in the T-cell infiltrate that was most marked for $CD4^+$ (T helper) cells and an increase in $CD1^+$ (Langerhans) cells in the epidermis after treatment. The epidermal defect with acantholysis was still present after clinical resolution of lesions and *in vitro* epidermal tissue cultures from affected patients reveal similar changes when removed from dermal influences.[8,9] We postulate a basic defect in cell adhesion throughout the skin of these patients. The disease only manifests when external factors such as friction, occlusion or infection provoke cytokine release and a lymphocytic inflammatory response.

The 'dilapidated brick wall' appearance seen on histology may offer a very easy route for the penetration of CyA and yet we were unable to produce a response by this route. Our experience suggests that this drug merits further evaluation in patients with severe and recalcitrant Hailey–Hailey disease.

Department of Dermatology, A.D.Ormerod
Aberdeen Royal Infirmary, Aberdeen AB9 2ZB; Janet Duncan*
**Department of Pathology,* L.Stankler
University of Aberdeen,
Aberdeen,
U.K.

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ADONIS 000709639100070N

Rothmund–Thomson syndrome

Sir, We read the recent case reports and review of Rothmund–Thomson syndrome with interest.[1]

In 1988 we presented two Turkish Cypriot women with the Rothmund–Thomson syndrome to the Section of Dermatology, The Royal Society of Medicine. They were the daughters of unrelated parents and, in addition to typical cutaneous features, had immunodeficiency, bronchiectasis and raised cholesterol and triglyceride levels. The older sister, in addition to low immunoglobulins, had low isohaemoglutannins as well as neutropenia and beta thalassaemia trait. They had no bone defects and DNA repair synthesis was normal. There was no evidence of hypogonadism—the older sister had had one normal child and a miscarriage.

The older sister, then aged 26, developed an acute myeloid leukaemia shortly after the case presentation and died within a few weeks, despite intensive therapy at the Royal Free Hospital.

As Moss has noted, associated malignancy and immune defects are unusual in the Rothmund–Thomson syndrome and may be simply coincidental. However, the association of these two rare conditions in our patient makes us feel that it may be of some significance and worthy of note.

Department of Dermatology, N.P.J.Walker
Whittington Hospital, C.M.Ridley
London N19 5NF, U.K.

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ADONIS 0007096391000712

Drug interactions with methotrexate

Sir, Zachariae[1] in his review of methotrexate side-effects states that interactions can occur with other drugs but 'in practice the only serious problems arise with the concomitant use of sulphonamides, especially if combined with trimethoprim, and salicylates'. This advice is incorrect and dangerous.

Drug interactions between methotrexate and non-steroidal anti-inflammatory drugs (NSAIDs), commonly used for the treatment of psoriatic arthropathy, are extremely important. I became aware of this problem when one of my patients who had received methotrexate 25 mg weekly for 4 years without ill-effect was given azapropazone for joint pains by her general practitioner and 2 weeks later developed life-threatening marrow suppression with mouth and genital ulceration.[2] My patient recovered, but others have been less fortunate, and there are now several reports of fatal or near-fatal interactions between methotrexate and various NSAIDs.[3–8]

All doctors who use methotrexate need to be aware of this dangerous interaction and the dose of methotrexate should be greatly reduced before NSAIDs are introduced.

Department of Dermatology,
Bristol Royal Infirmary,
Bristol BS2 8HW, U.K.
J.L.BURTON

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ADONIS 000709639100072F

Serum lipid elevation during isotretinoin therapy for acne in the west of Scotland

SIR, To support their contention that triglyceride levels continue to rise late in isotretinoin treatment of acne,[1] Dr Walker and Professor MacKie emphasize a comparison of maximum increases in serum triglyceride concentration between 40 patients treated for more than 16 weeks and 45 treated for 16 weeks exactly. However, it is stated elsewhere that only 36 samples were taken after 14 weeks, so unless there is a numerical error it seems samples taken before 16 weeks have been included in the data for the longer treatment group. These have no place in the comparison; in any case, if the group treated for longer had a greater number of samplings, selection of the highest value of each individual series would bias the result upward even in otherwise identical populations. The data in Table 1 suggests that after 14 weeks the retinoid-induced increase in triglyceride is, if anything, less. Figure 2 confirms that, with time, abnormal fasting triglyceride values may fall as well as rise. In one of the two subjects with worryingly high (>4 mmol/l) triglyceride levels treatment was continued; a later sample was normal.

A more appropriate statistical approach to studying the relationship between time and triglyceride concentration would be the use of within-subject comparisons. In a large 20-week study[2] which used such a method, triglyceride concentrations were maximal at 2 weeks in males; in females the major increase was at 2 weeks and an additional rise in weeks 8–12 was of lesser magnitude. Other studies also show that the major effect on serum triglyceride occurs within 4 weeks.[3] It seems premature to accept the authors' recommendation that, after 6 weeks, monthly measurement of serum triglycerides is necessary in this group of patients.

Department of Dermatology,
Royal Victoria Infirmary,
Newcastle upon Tyne NE1 4LP, UK.
**Department of Dermatology,*
The General Hospital,
Steelhouse Lane,
Birmingham B4 6NH, U.K.
C.S.MUNRO
J.R.MARSDEN*

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Reply

SIR, Drs Munro and Marsden dispute the validity of our conclusion that monitoring of serum triglyceride should be continued at monthly intervals during a course of therapy with isotretinoin. We accept that considering the population as a whole, there is no statistical confirmation from our own or previous studies[1,2] that elevations of triglyceride occurring after the first 6 weeks of therapy are significantly different from those occurring during the first 6 weeks, though it was a consistent observation in male patients studied by Zech *et al.*,[1] and in the female patients in the study by Bershad *et al.*,[2] that the mean value of triglycerides was higher in later samples than it had been at 2–6 weeks of therapy. We also accept the limitations of our data in Table 2 in which we chose to compare the maximal elevation of lipids occurring at any time during therapy in a group treated for exactly 16 weeks against a group treated for longer. We did not intend to over emphasize this result given that it did not achieve statistical significance.

However, the clinical question we wish to address cannot be answered by statistical comparison of the whole group, even using paired data. We need to know whether measurement of lipids within the first 6 weeks of therapy is a satisfactory predictor of any future increases in the individual patient. It was with this in mind that we interpreted Figures 1 and 2. We concluded that triglyceride levels were seen to rise to a dangerous level in some individuals when the value at 2–6 weeks of therapy was within the normal range. Drs Marsden and Munro emphasize the poor predictive value of triglyceride levels measured at 2–6 weeks by pointing out that levels may fall as well as rise thereafter, but it is because of this that one-way paired statistical analysis may not reveal any change in the population. These unpredictable changes in triglyceride levels in the individual patient, and the potential acute risks of elevations of triglyceride, led us to propose that repeated estimation of triglyceride during a course of therapy is valuable.

Department of Dermatology,
Anderson College Building,
56 Dumbarton Rd,
Glasgow G11 6NU, U.K.

B.R.Walker
Rona M.MacKie

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ADONIS 000709639100073S

The use of sunscreens in preventing skin cancer

Sir, A recent paper on senescence and sunscreens,[1] in our opinion, provides misleading and potentially harmful information for patients exposed to UV light. It is suggested that the daily use of a sun protection factor (SPF) 2 sunscreen will reduce the risk of skin cancer more effectively than those products with a high SPF value. This has no basis in fact. In one of the studies cited[2] both an SPF 2 and an SPF 15 product were tested and the results showed that the SPF 2 product reduced skin cancer as expected, while the SPF 15 product eliminated the risk entirely. In every other study conducted to date high SPF sunscreens were more effective in preventing photo-induced skin cancer.

The author mistakenly assumes that the application of a high SPF sunscreen will force users to increase their UV exposure. This assumption is contrary to the demographic data, which shows that individuals who intentionally expose themselves to long periods of sunlight in order to tan tend to use lower SPF sunscreens or no sunscreen at all. Conversely, sun-concerned individuals avoid excess sun exposure and use high SPF sunscreens.

The statement that sunscreens 'usually act as UVB (280–315 nm) filters' is incorrect. The SPF is a measure of the sunscreen product's effectiveness in preventing UV-induced erythema caused by both UVB and UVA. It is, in fact, impossible to develop a high SPF sunscreen ($\geqslant 15$) by only blocking UVB. Sunscreens with SPF values up to 45 have been developed which block most of the UVB and UVA spectrum.

The professional readership of the journal must give reliable information to their patients, and it would be irresponsible to suggest that less UV protection is better in the face of clear evidence to the contrary.

Consumer Operations,
Schering-Plough Corporation,
3030 Jackson Avenue,
Memphis,
TN 38151, U.S.A.

F.J.Akin
R.M.Sayre

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Reply

Sir, In response to the letter of Akin and Sayre, I regret that my English does not travel well. I certainly did not say, or even imply, that high SPF sunscreen will 'force users to increase their UVR exposure'. I said 'high SPF sunscreens may enable, or at least encourage, the user to spend more time in the sun'. There is more than a shade of difference between these statements and I fully accept that if behavioural patterns are not modified by sunscreen use, a high SPF preparation (e.g. 15) will offer more protection against cumulative UVR exposure than a low one (SPF 2). However, assuming that SPF = 1/effective transmittance,[1] the relationship between SPF and UVR absorbance is non-linear and shows diminishing returns with SPF $>$ 2. Thus, under comparable conditions, an SPF of 2 will absorb 50% UVR, an SPF of 4 will absorb 75%, an SPF of 8 will absorb 87% and an SPF of 15 will absorb 93%. As the active ingredients are probably the most costly components of a sunscreen, and cost is a probable determinant of use, I would suggest that an SPF of say 4 would be much more cost-effective than an SPF of 15 for prophylactic long-term non-beach use. Of course the optimum SPF will depend on the likely degree of exposure (e.g. U.K. vs. Tennessee).

Neither did I say that sunscreens 'usually act as UVB (280–315 nm) filters'. I said that 'The efficacy of such preparations (sunscreens) in preventing erythema is indicated by their SPF and their active agents (in preventing erythema in standard SPF tests) are usually UVB-(280–315 nm) absorbing molecules'. I recognize that many preparations contain UVA filters. However, most FDA-approved UVA-absorbing sunscreen agents do not have very impressive broad-band UVA absorption spectra. Furthermore, the UVA protection factors measured by Diffey and Robson[2] are generally low when compared with a given product's UVB protection factors.

Photobiology Unit,
Institute of Dermatology,
Lambeth Hospital,
London SE11 4TH, U.K.

A.R.Young

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ADONIS 0007096391000747

A Belgian case of the eosinophilia–myalgia syndrome

SIR, The association of oral ingestion of L-tryptophan with an unusual syndrome of eosinophilia and myalgia occurring in three patients in New Mexico in October 1989 led to the discovery of an epidemic of eosinophilia–myalgia syndrome (EMS).[1,2] By February 1990, 1321 cases of EMS had been reported with 15 deaths, mostly due to an ascending polyneuropathy that resembled the Guillain–Barré syndrome and from respiratory failure. Cerebrovascular accidents have also been reported.[1,3-5] We report a Belgian case of EMS.

A 52-year-old man in February 1990 developed muscular aches and weakness. He had also a burning sensation in his skin. The myalgia got progressively worse and was associated with a generalized rash of erythematous macules with a purple hue (Fig. 1). A reticulate pattern was observed on the lateral side of the upper arms. The rash was most pronounced on the shoulders and thighs. The patient had been taking L-tryptophan, 3–6 g every evening, for the previous 1·5 years for insomnia. Other medications included triazolam, 0·75 mg/day and multivitamins.

He was admitted to hospital on 12 March 1990 with incapacitating muscle pains. He had no history of Raynaud's phenomenon, arthralgia, respiratory symptoms and had no fever or other symptoms associated with connective tissue disorders.

Laboratory investigations showed a white cell count of $14{\cdot}6 \times 10^{9}$/l with 28·6% eosinophils and an absolute count of $4{\cdot}17 \times 10^{9}$/l. Bone-marrow examination revealed an eosinophilia of 17% but was otherwise unremarkable. The serum lactate dehydrogenase level was 609 U/l (normal range 200–450 U/l) with a relative increase of the LDH3 fraction. The serum aldolase level was 4·2 U/l (normal range 0·5–3·1 U/l) but the creatine kinase activity was depressed to 24 U/l (normal range 38–195 U/l). The erythrocyte sedimentation rate was 18 mm/h and the C-reactive protein was 22·3 mg/l.

Serum levels of IgG, IgM, IgA, C3 and C4 were all normal. Antinuclear antibodies were positive with a homogeneous pattern. Cryoglobulins, lupus anticoagulant, rheumatoid factor, LE-test and anti-DNA antibodies were all normal or negative. Electromyography and electroencephalography were carried out and were normal. A muscle biopsy revealed a discrete perivascular infiltrate of lymphocytes without vasculitis. The muscle cells appeared normal, the histology of a skin biopsy was non-specific and direct immunofluorescence did not reveal any deposition of immunoglobulins or complement. A chest X-ray and electrocardiogram as well as abdominal ultrasound and computerized tomography were all normal. Tests for ova and parasites in the stools and for antibodies to *Ascaris, Echinococcus, Toxocara, Trichinella*, hepatitis B virus, *Toxoplasma* and *Cytomegalovirus* were negative.

A diagnosis of eosinophilia–myalgia syndrome was made and the patient was advised to stop taking L-tryptophan. Four weeks later his total eosinophil count was $2{\cdot}85 \times 10^{9}$/l. He was then treated with the non-steroidal anti-inflammatory drug nifluminic acid.

Two months later the patient developed morphoea-like features involving the arms and legs. The skin was indurated and had a glistening, ivory-coloured surface (Fig. 2). There was no sclerodactyly, digital ulcerations or periungual telangiectasia and he had no systemic symptoms. The reflexes in the lower extremities were absent and he had paraesthesiae affecting both hands. Electromyography indicated a progressive axonal neuritis and a diagnosis of active axonal polyneuropathy was made. The histology of a further skin biopsy showed a moderate thickening of the dermis with a sparse lymphocytic infiltrate around the blood vessels. The fascia showed intense inflammation with lymphocytes and histiocytes and only a few

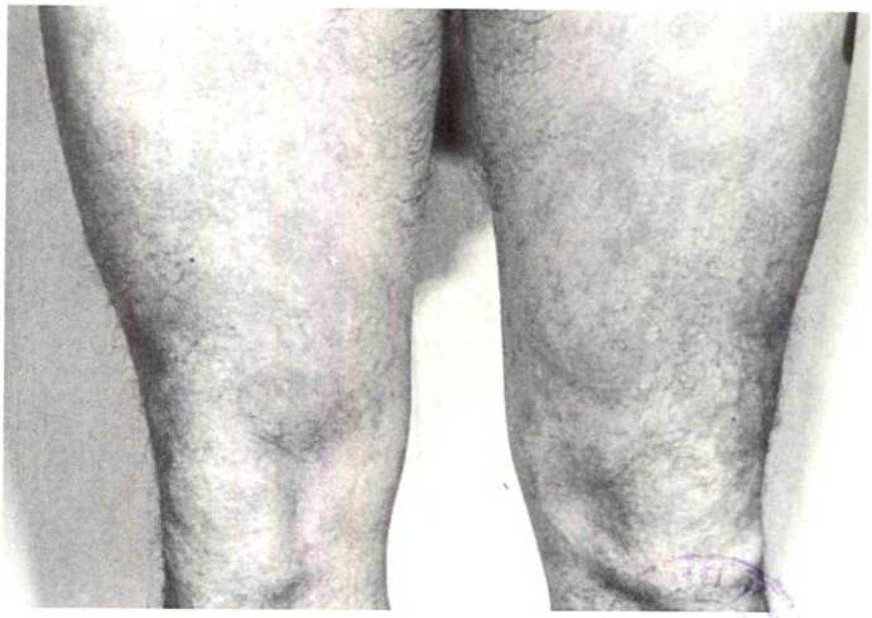

Figure 1. Erythematous macules on thighs.

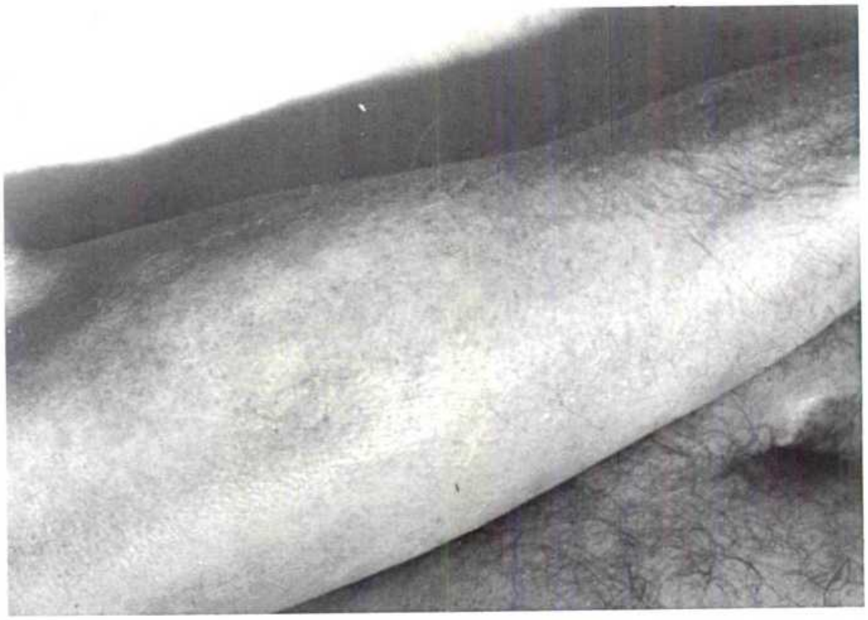

Figure 2. Shiny, ivory-coloured skin on forearms.

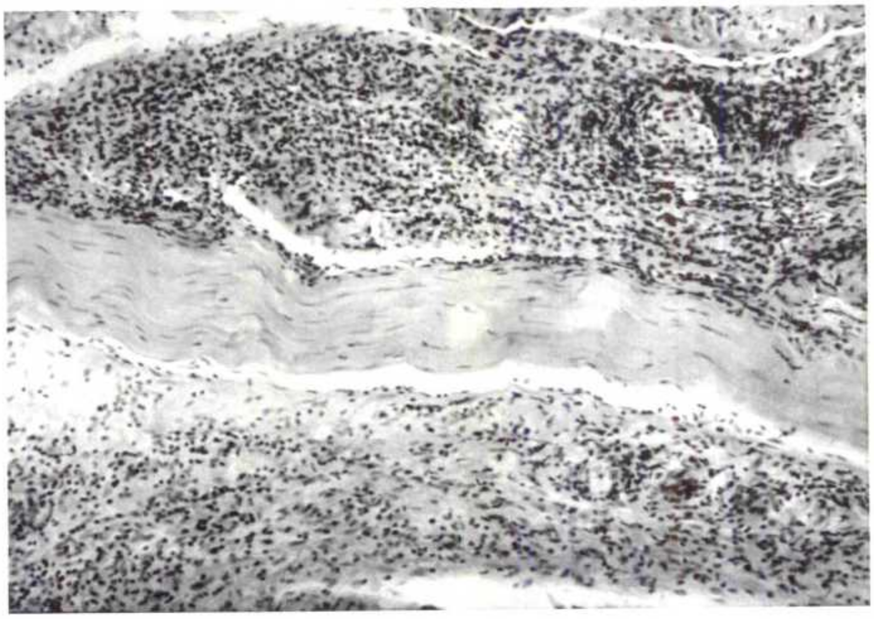

Figure 3. Marked lympho-histiocytic infiltration of the fascia.

eosinophils (Fig. 3). The absolute eosinophil count was $2{\cdot}41 \times 10^9$/l.

He was treated with prednisone at a dosage of 100 mg daily for 4 weeks with a subsequent tapering of the dose. The eosinophil count lowered dramatically to $0{\cdot}13 \times 10^9$/l, but the scleroderma-like features in the skin showed a much slower response. The patient is currently receiving 65 mg of prednisone daily and 100 mg azathioprine.

Our patient fulfils the criteria for EMS[3] and developed fasciitis and scleroderma-like features 2 months after stopping taking L-tryptophan. At the time of writing we are aware of eight other cases of EMS in Belgium (Belgian Centre for Pharmacotherapeutic Information). Most of the published cases of EMS originate in the U.S.A., but recently two French[6] and two Belgian cases[7] have been reported.

L-tryptophan has been prescribed in Belgium for insomnia, depression and for 'stress' for a number of years. The tryptophan taken by our patient originated at least in part from Japan where the major producer is located. The cause of EMS at present remains unknown, but it is likely to be due to a contaminant of the tryptophan during the production process and possibly abnormal tryptophan metabolism in susceptible individuals.[8] The skin is one of the important targets in EMS with transient maculopapular or urticarial rashes, alopecia and later on morphoea-like changes. Dermatologists should be aware of these signs in order to recognize this syndrome that can be potentially fatal.

Department of Dermatology,
University Hospital,
De Pintelaan 185,
00 Gent,
lgium

J.M.Naeyaert
C.Cuelenaere
J.De Bersaques
D.Platevoet
A.Kint

erences

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Book Reviews

Ozone Depletion. Health and Environmental Consequences. Edited by Robin Russell Jones and Tom Wigley (1989). Chichester: John Wiley and Sons. Pp. 280. ISBN 0-471-92316-8.

This book is the proceedings of an international conference held in November 1988. It summarizes much of the available scientific information on ozone depletion and the allied problem of global warming up to that date and, as stated in the preface, is unique in bringing together the views of physicists, medical scientists and clinicians, politicians and environmentalists. Despite this spectrum of expertise, the authors and editors have for the most part succeeded in producing chapters which are comprehensible without specialist knowledge, although readers without a science background would struggle in places.

The book is divided into sections which discuss recognition, measurement, control, and ecological and health consequences, of ozone depletion. Multiple authorship has inevitably led to some duplication of background information, but this is necessary as some readers will pick out specific areas of interest; a summary of the basics of ozone depletion is also given at the start of the book in a short but very useful 'fact sheet'.

Four chapters, about a quarter of the book, are specifically relevant to dermatologists. Arlett and Cole discuss photosensitive human syndromes, with data predominantly from cellular studies in xeroderma pigmentosum; the difficulty here is how far the results can be extrapolated to normal skin *in vivo*. In a chapter which is rather more comprehensible to the potentially non-dermatological readership, van der Leun presents a readable account of ultraviolet carcinogenesis and introduces the concepts of optical amplification factors (increase of biologically active ultraviolet due to decreased ozone layer) and biological amplification factors (increase in cancers due to increased ultraviolet dose). The epidemiology of skin cancers related to ultraviolet radiation forms the major part of a wide-ranging and well referenced chapter by Russell Jones, and Elwood reviews the epidemiology of malignant melanoma and gives a detailed discussion of the relationship with ultraviolet radiation.

The difficulties of prediction of the state of the ozone layer in the next century will produce a feeling of *déjà vu* for National Health Service employees; the subject is bedevilled by lack of funding, measurement systems of uncertain accuracy, and predictive models which produce disparate results. There are differences of opinion regarding the required stringency of control measures, and disagreement between politicians and environmental pressure groups regarding the achievements so far. It is an emotive topic and the media attention generated by each conference and publication means that it can be difficult to weed out the facts from the speculation; those who want to read beyond the newspaper headlines should read this book.

N.H.Cox

The Metabolic and Molecular Basis of Acquired Disease—Volumes 1 and 2. Edited by R.D.Cohen, B.Lewis, K.G.M.M.Alberti and A.M.Denman (1990). London: Bailliere Tindall. ISBN 0-7020-1282-3 (Vol. 1), ISBN 0-7020-1369-2 (Vol. 2), ISBN 0-7020-1493-1 (Set). Price £175.00.

This is a two-volume set comprising 2082 pages and in the preface states that it is enquiring about mechanisms of acquired disease at the biochemical and molecular level. Specific exclusions have been made of inborn errors of metabolism.

There are five main sections. The first deals with biochemical mechanisms of disease including basic concepts of carcinogenesis, immunological mechanisms, steroid receptors, etc. The second concerns environmental aspects of disease dealing with the physical, chemical and nutritional environment. Th third deals with acquired metabolic disease, the fourth wit metabolic and molecular aspects of 'non-metabolic' diseas and the last, which is a single section, is on human retrovi uses. The only two chapters specifically on dermatologica problems are one on hyperproliferative and immunologica diseases of the skin and one on ultraviolet radiation-induced skin disease which—rather strangely—is not found in the section on physical environment.

I think most dermatologists would choose to consult th book for the non-dermatological content as the dermatologic aspects are covered more fully elsewhere. I found the chapters on amyloid, on diabetes mellitus and on retroviruses particularly well done. In some of the other chapters the content and referencing is perhaps rather patchy.

At this time, when understanding of molecular mechanisms is advancing rapidly, it is likely that some sections of the book will be rapidly outdated. At present, however, it is a useful reference work although expensive at £175.

Rona M.MacKie

Sunscreens: Development, Evaluation, and Regulatory Aspects. Edited by N.J.Lowe and N.A.Shaath (1990). New York: Marcel Dekker Inc. Pp. 624. ISBN 0-8247-8265-8. Price $180.

With the increasing awareness of the need for protection against sunlight, this book, which aims to be a reference guide to sunscreens, has made a timely appearance. It is one in a series of volumes concerned with cosmetic science and technology, and is aimed at all those involved in the formulation, testing and use of sunscreening agents.

The book has six sections, three of which are concerned mainly with the chemical properties of sunscreens and their formulation. One section deals with regulatory aspects, and the remaining two sections cover photobiological aspects of sunscreens. The structure and photochemistry of sunscreens are described in detail, along with the techniques for formulation of sunscreen products and their quality control. A reference chapter gives very detailed spectroscopic data of the 17 most widely used sunscreen chemicals.

That the book is designed to appeal to an international readership is shown by chapters that review the sunscreen industries in the United States, Europe, Japan and Australia. The history and growth of the use of sunscreens in the United States and Australia are described particularly well, as are the complex regulations in the United States where sunscreens are regarded as drugs rather than cosmetics.

Sunscreen photobiology is covered in two sections: one reviews many of the biological effects of sunlight on the skin and the other is concerned with biological evaluation of sunscreen products. Measurement of sun protection factors, water resistance and problems with *in-vitro* testing of products are described in detail. Unfortunately much information is repeated, particularly with regard to UVA protection and its measurement, and the mechanism and importance of melanin pigmentation (which is described in three different chapters).

The use of 41 authors to write the 34 chapters in the book has led to much variation in style and quality (some chapters are presented in little more than note form), and much repetition. Nevertheless this book succeeds in its aim to be a reference guide to sunscreens, and contains much that will be of interest to dermatologists.

P.M.FARR

British Journal of Dermatology (1991) **124**, 308.

News and Notices

Melanoma '91, Brighton, 8–11 May 1991

Melanoma '91, the Brighton International Melanoma Conference, will be held at the Brighton International Conference Centre on 8–11 May 1991. This multidisciplinary meeting will address problems of the natural history, diagnosis, management and prevention of melanoma and will comprise keynote addresses, symposia, proffered papers, biopsy seminars and workshops in oncology, dermatology, pathology and surgery. The conference is organized by the Melanoma Study Group. For further details contact: Dr N.Kirkham, Department of Histopathology, Royal Sussex County Hospital, Brighton BN2 5BE. Telephone 0273-696955 ext. 4269, Fax 0273-600182.

11th Congress of the International Society for the Study of Vulvar Diseases, Oxford, U.K., 22–26 September 1991

The 11th Congress of the International Society for the Study of Vulvar Diseases will take place at the Randolph Hotel, Oxford on 22–26 September 1991. For further information please contact: Dr C.M.Ridley (President ISSVD), Whittington Hospital, London N19 5NF. Telephone: 071-272-3070 ext 4575. Following the Congress there will be a *Study Day on 27 September* in conjunction with the Medical Society for the Study of Venereal Diseases, and speakers from both societies will cover topics of common interest. For further information please contact: Dr C.M.Ridley (at above address) or Dr M.J.Godley (Honorary Secretary MSSVD), The Royal Berkshire Hospital, Reading RG1 5AN. Telephone 0734-877206.

Institute of Dermatology, United Medical and Dental Schools, University of London

On 8 October 1990 Her Royal Highness the Princess Royal, Chancellor of the University of London, unveiled a plaque to mark the inauguration of the Institute's new premises at St Thomas' Hospital.

Notice to Contributors

The *British Journal of Dermatology* publishes original articles on all aspects of the normal biology, and of the pathology of the skin. Originally the Journal, founded in 1888, was devoted almost exclusively to the interests of the dermatologist in clinical practice. However, the rapid development, during the past twenty years, of research on the physiology and experimental pathology of the skin has been reflected in the contents of the Journal, which now provides a vehicle for the publication of both experimental and clinical research and serves equally the laboratory worker and the clinician. To bridge the gap between laboratory and clinic the Journal publishes signed editorials reviewing, primarily for the benefit of the clinician, advances in laboratory research, and also reviews of recent advances in such aspects of dermatology as contact dermatitis, therapeutics and drug reactions. Other regular features include book reviews, correspondence and society proceedings. The Journal is the official organ of the British Association of Dermatologists and also of the Netherlands Association for Dermatology and Venereology, but it attracts contributions from all countries in which sound research is carried out, and its circulation is equally international.

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kilogram(s)	kg
milligram(s) (10^{-3}g)	mg
microgram(s) (10^{-6}g)	μg
nanogram(s) (10^{-9}g)	ng
picogram(s) (10^{-12}g)	pg
hours(s)	h
minute(s)	min
centimetre(s)	cm
second(s)	s
cubic millimetre(s)	mm^3
millimetre(s)	mm
millicurie(s)	mCi
milliequivalent	mmol
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millilitre(s)	ml
gravitational acceleration	*g*
micrometre(s)	μm
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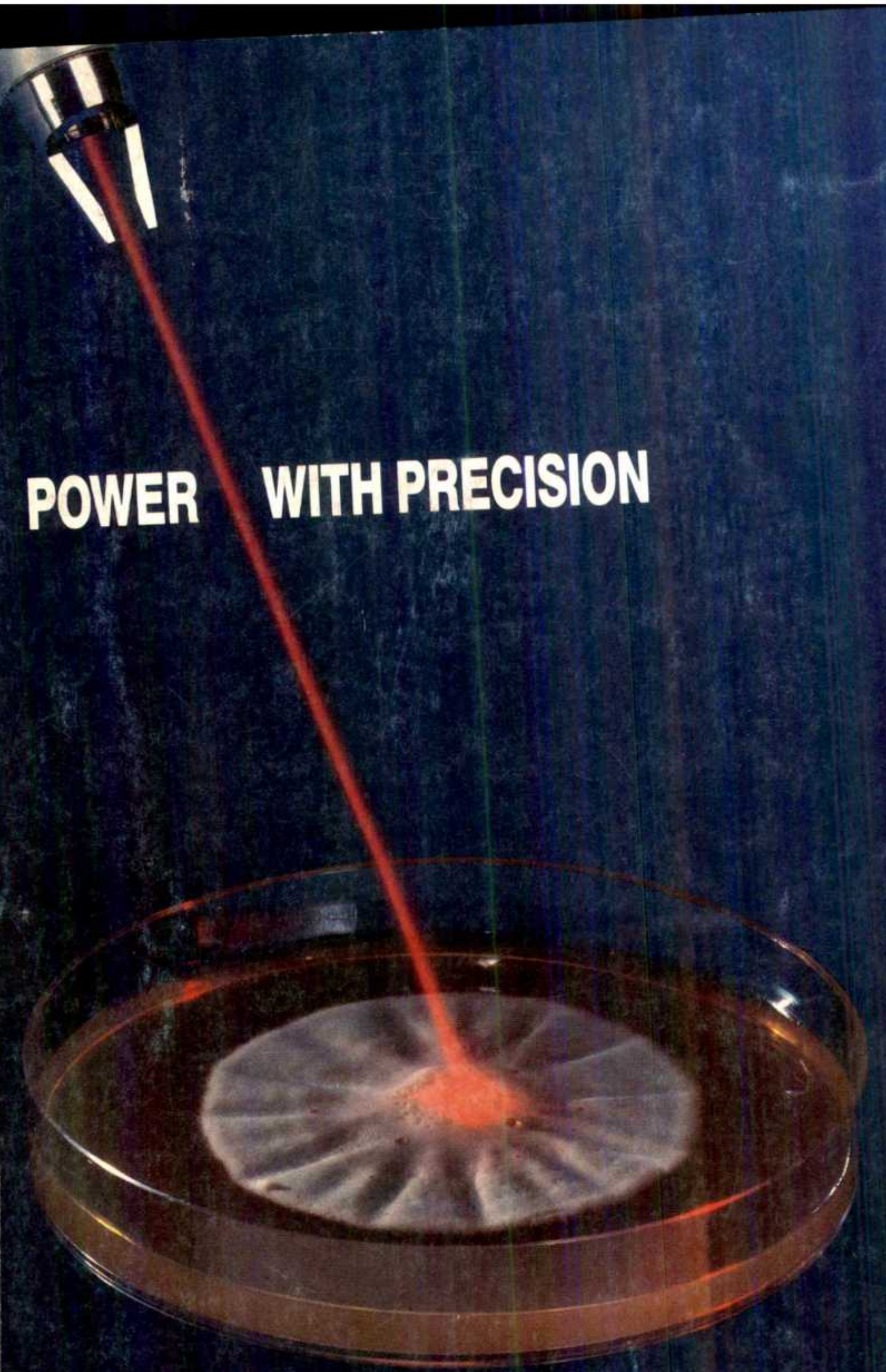

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